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Full Length Research Paper

Control of *Sclerotium rolfsii* in peanut by using *Cymbopogon martinii* essential oil

Yrlânia de Lira Guerra[1], Thiago Alves Santos de Oliveira[1], Delson Laranjeira[1], Liziane Maria Lima[2], Péricles de Albuquerque Melo Filho[1] and Roseane Cavalcanti dos Santos[2]*

[1]Plant Pathology, Rural Federal University of Pernambuco, Recife-PE, Brazil.
[2]Biotechnology Laboratory, Embrapa Cotton, Campina Grande-PB, Brazil.

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Essential oils of seven species were investigated in order to control peanut plants against white mold (*Sclerotium rolfsii* Sacc.). The assays were carried out by *in vitro* and *in vivo* assays. At first, fungitoxicity and suppression of oxalic acid diffusion (SOAD) bioassays were performed in order to evaluate the mycelial growth of fungus. Then, validation assays were carried out in greenhouse, involving inoculation of fungus in the seeds and further plant treatments with essential oil. Four isolates of *S. rolfsii* were tested in different oil concentrations. *Cymbopogon martinii* oil at 300 ppm inhibited the mycelia growth of *S. rolfsii* in 55% and also the number of sclerotia. In validation assay, we found that a single dose of *C. martinii* oil at 400 ppm reduced the rate of disease in 55%, confirming the *in vitro* assays. The follows traits: number of pods/plant, pod weight and harvest index increased in 57, 54, and 40%, respectively, in all *C. martini* oil treatments. These results demonstrate that *C. martinii* oil at low concentration may serve for new formulations in the treatment and prevention of white mold.

Key words: *Arachis hypogaea,* disease control, toxicity, white mold.

INTRODUCTION

Peanut (*Arachis hypogaea* L.) is one of the world's most important oleaginous, grown widely to attend the edible oil and food markets. More than half of the production area of peanut fall under arid and semi-arid regions, where peanuts are frequently prone to drought stresses (Reddy et al., 2003). In addition, drought conditions influence the growth of weeds, agronomic management and, nature and intensity of pests, including insects, weeds and diseases (Staley et al., 2006).

Diseases caused by fungus are a serious problem to peanut crop. Annually, large amounts of fungicides are sprayed in field in order to control leaf and soil fungus. The indiscriminate use of chemical pesticides has given rise to several problems, such as genetic resistance of pest species, toxic residues in stored products, increasing costs of application, hazards from handling, environmental pollution, and others (Adeyemi, 2010). Genetic resistance to diseases is a main goal in breeding

*Corresponding author. E-mail: roseane.santos@embrapa.br. Tel: + 55 83 31824300.

Table 1. *Sclerotium rolfsii* isolates used in the pathogenicity assay.

Isolate	Code	Access	Local	Host	Lat/Long	Botanic specie
SR5	S.r. 28	LEDP	Cristalina, GO	chickpea	18°10'12"S,47°56'31"W	*Cicer arietinum*
SR14	CMM 2115	CMM	Teresina, PI	cowpea	5°5′20″S,42°48′7″W	*Vigna unguiculata*
SR15	CMM 2930	CMM	Potengi, CE	cowpea	7°5′27″S,40°1′37″W	*V. unguiculata*
SR16	CMM 3051	CMM	Alhandra, PB	cowpea	7°26′20″S,34°54′50″W	*V. unguiculata*

programs, however, depending on the pathogen, the progress is limited due to lack of resistant germplasm.

White mold, caused by *Sclerotium rolfsii* Sacc., is a dangerous pathogen of several crops and is found throughout the major crop areas in many countries, causing yield losses >40% in peanut, bean, garlic, onion, and pepper plant (Fery and Dukes, 2011; Adandonon et al., 2006; Earnshaw et al., 2000). In Brazil, no commercial cultivar of peanut has resistance to white mold, so that management in areas infested with the fungus is often hampered due to limitations to control the disease. Moreover, as inoculum has high persistence in soil, the eradication of pathogen is low efficient and quite expensive (Ozgonen et al., 2010; Punja, 1985). The cost to chemical treatment of the seeds burdens the production system, besides environmental damages caused by pesticide residues. Besides, as resistance development is a real problem faced by the indiscriminate use of synthetic pesticides, it is likely that the protection of plants by biopesticides will be more durable due to various components contained in extracts or essential oils (Koul et al., 2008).

Several metabolities have been reported as effective biopesticides against various species of phytopathogens, highlighting the essential oils that contain up to 60 distinct chemicals, with more than two main components (Hillen et al., 2012; Abdolahi et al., 2010; Bajpai and Kang, 2010; Bakkali et al., 2008). The toxicity of these oils is more related to phenolic compounds and terpenoids, that have high antimicrobial activity and are found in several plants such as lemon grass (*Cymbopogon* sp.), *Eucalyptus* sp., rosemary (*Rosemarinus sp.*), vetiver (*Vetiveria sp.*), clove (*Eugenia* sp.), thyme (*Thymus* sp.), and others (Melo et al., 2013; Das et al., 2010; Vukovic et al., 2007). In fungus, Chen and Viljoen (2010) report that antimicrobial action of *Cimbopogon* oil involves the passive entry of the oil into the plasma membrane in order to initiate membrane disruption, and after to inhibit the cell growth due to accumulation in the plasma membrane. The bilayer disorder and ion leakage disturb the osmotic balance of the cell through loss of ions. Full inhibition of mycelia growth and spore germination have been demonstrated in *Phakopsora pachyrhizi*, *Colletotrichum gloeosporioides*, *Didymella bryoniae,* *Cladosporium* sp., *Nigrospora* sp. and others (Souza Junior et al., 2009; Mata et al., 2009; Medice et al., 2007; Fiori et al., 2000).

The present work was proposed in order to investigate the antifungal activity of different essential oils to control peanut plants against *S. rolfsii*, based *in vitro* and *in vivo* assays.

MATERIALS AND METHODS

Origin of *S. rolfsii* isolates and disease severity assay

Four *S. rolfsii* isolates (Table 1) were kindly supplied by the Maria Menezes Collection, from Rural Federal University of Pernambuco (UFRPE), Brazil. Previous pathogenicity assays were carried out in greenhouse, using peanut plants, in order to estimating the disease severity, following methodology described in Bastos and Albuquerque (2004).

The isolates were previously grown on autoclaved rice during nine days in Petri dishes and further added to a commercial substrate (Baseplant) at 72 mg.kg^{-1}, in pots (1 L) (Barbosa et al., 2010). Three peanut seeds previously surface-sterilized (hypochlorite solution at 1.5%) were sown in each pot and daily watered. Two earliness-upright cultivars were used in this assay: Senegal 55 437, a Spanish type developed by International Crops Research Institute for the Semi-Arid-Tropics (ICRISAT), and BR 1, a Valencia type developed by Brazilian Company of Agricultural Research (EMBRAPA). Taking in account that the low fertility of substrate could affect the fungus pathogenicity, this assay was repeated in the same conditions with supplementation of 40 g P_2O_5, 15 g KCl and 200 g hummus, added to each kg substrate, based on recommendations in Santos et al. (2006). The experimental design was completely randomized with eight replications.

Plants were monitored daily for 15 days to follow pathogen establishment and development of disease symptoms. The disease severity (DS) was evaluated following the scale described by Fery and Dukes (2002) (that is, 1 = no wilting symptoms, 2 = slight or partial wilting, 3 = general plant wilting, 4 = permanent wilt, and 5 = dead plant). Then, the disease severity index (DSI) was estimated on the basis of this rating scale by adopting the following formula

(Galanihe et al., 2004): $\text{DSI (\%)} = \sum \frac{(P \times Q)}{(M \times N)} \times 100$

Where, P = severity score, Q = number of infected plants showing the same score, M = total number of observed plants, and N = maximum rating scale.

Inhibition bioassays with essential oils *in vitro*

Seven pure essential oils, obtained commercially, were used in this assay: *Cymbopogon martinii* (Roxb.) Stapf var. *motia* Burk (Accession 1), *Cedrus atlantica* Manetti (Accession 2), *Copaifera officinalis* L (Accession 3), *Zingiber officinale* L (Accession 4), *Eucalyptus staigeriana* F. (Muell) (Accession 5),. *Juniperus communis* L. (Accession 6), and. *Ocimum basilicum* L. (Accession 7).

Oils were added separately to potato dextrose agar (PDA) culture

medium at 50°C and poured onto Petri dishes (9 cm diameter). A 0.5 cm-PDA disk containing mycelium from each isolate was deposited in the center of each plate (Melo et al., 2013). The negative control was oil-free. Then, plates were randomized and incubated in a biochemical oxygen demand (BOD) growth chamber at 28°C and 12:12 h photoperiod. The bioassay was completely randomized with seven replications for each concentration. Thereafter, the number of the sclerotia were counted in each treatment every 24 h for 15 days.

Initially, all oils were previously bioassayed at 1500 ppm with *Sclerotium*-isolates in order to evaluating the mycelia growth inhibition. Then, a new screening was performed at low concentrations (300, 400, 500, 600, 700, 800, and 900 ppm) using only the oils that showed initiation of mycelia growth until 1500 ppm. In both bioassays, the experimental design was completely randomized with ten replications.

Suppression of oxalic acid diffusion (SOAD) bioassay

A 523 medium (Kado and Heskett, 1970), supplemented with streptomycin sulfate (150 ppm), penicillin G (150 ppm), and bromophenol blue (150 ppm), was utilized for SOAD bioassay. The pH was adjusted to 4.7 (adapted by Steadman et al., 1994). Essential oils were added to the medium at the lowest inhibiting concentration verified by previous bioassays. A 0.5-cm-diameter disk of the PDA medium with a five-day mycelium was deposited in the center of each plate and incubated in the BOD growth chamber at 28°C for a 12:12 h photoperiod. The negative control was oil-free. The bioassay was completely randomized with five replications. The capacity of each oil to alkalinize the medium was evaluated by restricting the oxalic acid diffusion produced by the pathogen, which was visualized by the formation of a yellow halo of inhibition. The measurements were taken from the diameter of the halo.

Validation assay of peanut protection against *S. rolfsii* greenhouse

Based on bioassay results, a validation assay was performed in order to test the effectiveness of the essential oils against *S. rolfsii* in greenhouse. The assay was carried out in conditions adjusted to 77-86% relative humidity and 39–45°C air-temperature.

Although no report of germination inhibition of peanut seeds due to use of essential oils has been found, a preliminary germination test was conducted with 100 peanut seeds using essential oils at 1000 ppm, in growth chamber during seven days. All seeds have normal germination and no occurrence of toxicity was found (data not shown).

Seeds of the cv. BR 1 were sown in pots (5 kg) containing commercial substrate (Baseplant) supplemented with 40 g P_2O_5 + 15 g KCl + 200 g of humus per kg of substrate. *S. rolfsii* was added to substrate at 72 $mg.kg^{-1}$. Three peanut seeds previously surface-sterilized (hypochlorite solution at 1.5%) were sown in each pot and after 15 days, just two plants were remained. Normal watering was maintained throughout trial.

The follows treatments were evaluated: NC, negative control (seed treated with water, oil-free), PC, positive control (seeds previously treated with commercial fungicide, oil-free), ST, seeds previously treated with essential oil at 400 ppm, ST/11- ibid + 11 weekly applications of oil at the same concentration, ST/9- ibid + nine applications of decennial oil at the same concentration, ST/6- ibid + six biweekly applications of oil at the same concentration, and ST/3- ibid + three monthly applications of oil at the same concentration.

In PC-treatment, a fungicide based on Carboxin + Thiram (250 mL/100 kg of seed) was used. To ST-treatment, seeds were kept for 30 min in contact with the oil and then were sown; in the others treatments involving spraying, the oil was mixed in the irrigation water.

The completely randomized design was adopted with five replications. At the harvest, the disease severity was estimated according to the scale described by Fery and Dukes (2002). The traits pod weight, number of pods/plant, and the harvest index index was also estimated. Harvest index (HI) was calculated as total pod yield/total biomass including pod weight at final harvest (Nigam et al., 2005).

Statistical analysis

Data were submitted to analysis of variance using Statistix (version 9.0). The Tukey test ($p < 0.05$) was used for average comparisons. Data from the DSI were previously tested to normality according to Shapiro-Wilk test and further transformed using the function [$\sqrt{(x + 0.5)}$].

RESULTS AND DISCUSSION

Four *S. rolfsii* isolates were bioassayed as to pathogenicity assays in peanut plants, in greenhouse. The first symptoms were verified 48 h after inoculation, which evolved to stem bottleneck and plant wilting with the presence of white mycelium. The disease severity index (DSI) caused by *S. rolfsii* in two peanut cultivars is shown in Table 2. The pathogenicity of isolates was more pronounced in plants grown in fertilized soil. Based on scale reported by Fery and Dukes (2002), the isolate SR5 showed high severity. Therefore, it was chosen for further assays.

The cv. BR 1 showed high sensitivity to *S. rolfsii* isolates (Figure 1). The DSI ranged from 26 to 98% when plants were grown in substrate and from 20 to 76%, in soil with fertilizer supplementation. The relative differences in disease severity due to fertilizer supplementation ranged from 22 to 67%, for BR 1, and 67 to 94%, to Senegal for 55 437, indicating that although BR 1 is more sensitive to the pathogen, fertilization contributed to alleviate the effect of the disease. These results confirm the findings in the literature that the incidence of infection caused by *S. rolfsii* is reduced in well-nourished plants (Basseto et al., 2007; Mascarenhas et al., 2003). Based on pathogenicity assays the isolate *S. rolfsii* - SR5 and the sensitive cv. BR 1 were chosen to further validation assay.

Up till now, no report of tolerance to *S. rolfsii* is Brazilian fields involving peanut commercial cultivar is found. Based on low DSI seen in Senegal 55437 (Table 2), we suggest that it may be a genetic resource with tolerance to white mold and further studies should be encouraged to attest that suggestion. The low severity of disease may be associated with high earliness and short cycle (only 75-80 days), limiting a fast spreading of fungus, especially in reproductive phase (Duarte et al., 2013; Boote and Hammond, 1981).

Mycelia inhibition of *S. rolfsii* with essential oils

These bioassays were carried out with SR5 grown in

Table 2. Disease severity index in peanut cultivars inoculated with *Sclerotium rolfsii* isolates.

Treatment	BR1		RD (%)	Senegal 55437		RD (%)
				RD (%)		
	S	S+F		S	S+F	
Control	0dA	0dA	-	0eA	0cA	-
SR5	98aA	76aB	22	24aA	8aB	67
SR14	76bA	36bB	53	14bA	4bB	71
SR15	72bA	24cB	67	4cdA	0.5cB	87
SR16	26cA	20cA	-	8cA	0.5cB	94
Coefficient of variation (%): 14.38						
General average: 5.14						
Standart error: 0.49						
Treatment square mean: 64.54 Freedom degree: 4						
F test: 117.94						

S, Substrate without fertilizer supplementation; S + F, with fertilizer supplementation. RD, relative difference in disease severity based on fertilizer supplementation. Original data transformed by √ (x + 0.5) for statistical analysis. Means with the same letters do not differ statistically by Tukey test ($p < 0.05$). Letters on the line (capitals) represent among-treatment comparisons; letters in the columns (lowercase) represent among-isolate comparisons.

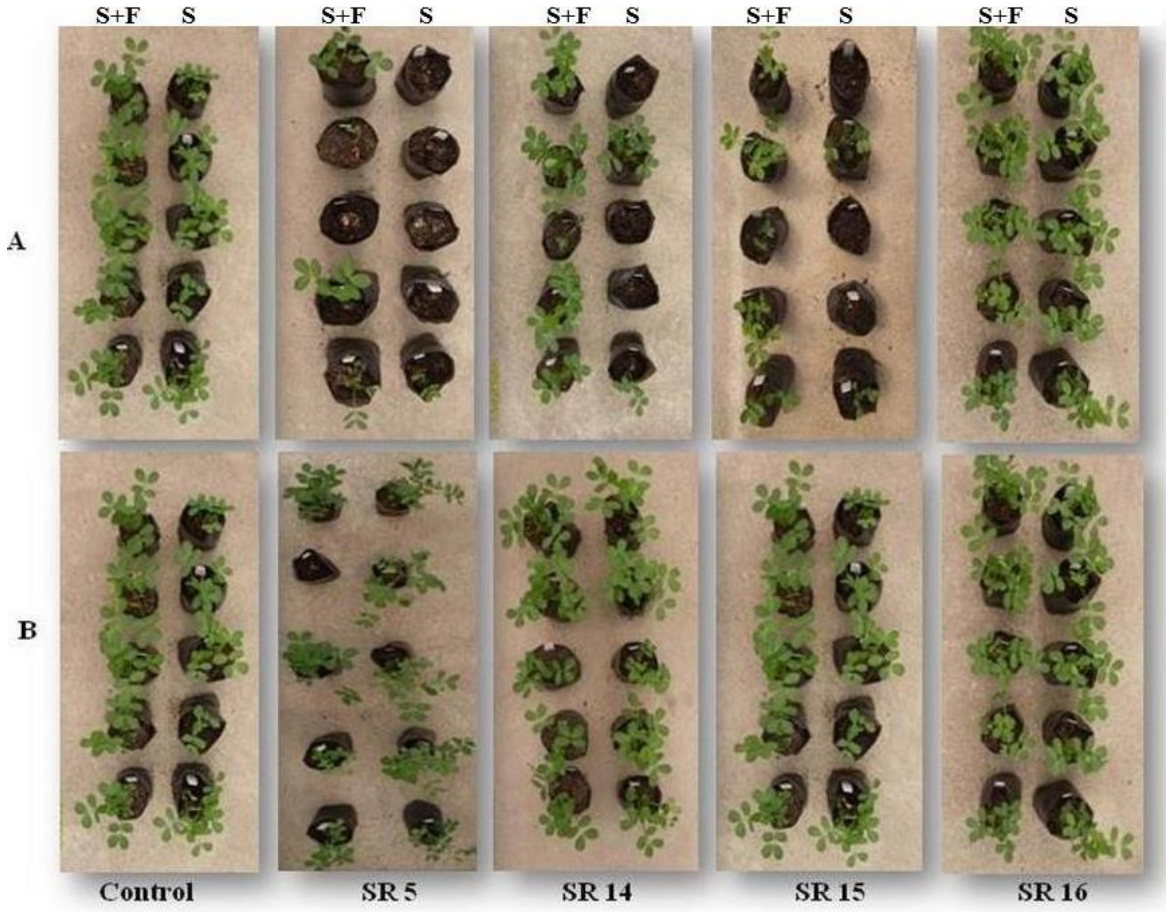

Figure 1. Pathogenicity assays in peanut plants carried out in greenhouse. **A.** BR 1. **B.** Senegal 55 437, S-Substrate, S+F- Substrate + fertilizer.

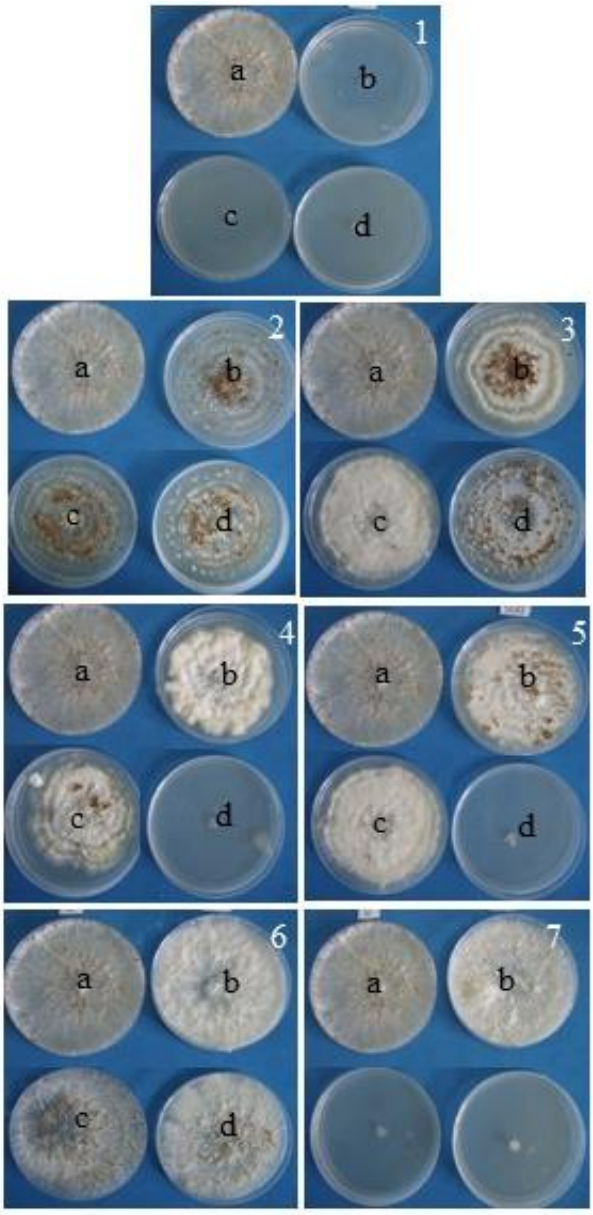

Figure 2. Inhibition of mycelial growth in SR5 *S. rolfsii* grown in (PDA) with several concentrations of essential oils; 1. *Cymbopogon martini*; 2. *Cedrus atlantic;* 3. *Copaifera officinalis*; 4. *Zingiber officinale;* 5. *Eucalyptus staigeriana*; 6. *Juniperus communis*; 7. *Ocimum basilicum*; concentrations were a. control (PDA), b. 500 ppm, c. 1000 ppm, and d. 1500 ppm.

PDA+ essential oils of seven species, at first at 1500 ppm, and then at low concentrations. Only *C. martinii* (Accession 1) inhibited mycelia growth (Figure 2) and sclerotia number (Table 3) in all concentrations. Therefore, oil from Accession 1 was chosen for further assays.

The antimicrobial action of *C. martinii* oil have been reported against several leave and soil pathogens, such as *Alternaria* sp., *Rhizoctonia solani, Aspergillus* sp., *Colletotrichum* sp., *Botrytis cinerea*, and others (Hillen et al., 2012; Stangarlin et al., 2011; Misra et al., 1988).

The biopesticide activity is mainly attributed to citronelal, geraniol and citronelol contents that also exhibit insecticide and nematicide effects (Barros et al., 2009; Hierro et al., 2004; Labinas and Cromo, 2002; Misra et al., 1988). *S. rolfsii* is a soil-born fungus, whose control is quite difficult and expensive. The possibility of control via no-chemical fungicide provides a reasonable perspective of healthy management to several host crops. Some reports have evidenced the control of white mold by using essential oil from *Origanum syriacum* L., *Foeniculum vulgare* Mill. and *Laurus nobilis* L. Mahato et al. (2014) evaluated the sensitivity of *S. rolfsii* towards some fungicides and botanicals and found that the inhibitory effects of different fungicides, essential oils and plant extracts are quite similar, situating at 86 to 95%.

Suppression of oxalic acid diffusion (SOAD)

Although no sclerotia was found at 300 ppm in isolate SP5, SOAD was performed with *C. martinii* oil at 400 ppm, taking in account a reliable safety margin for further recommendation. In this condition, no mycelia or sclerotia were found (Figure 3). The mycelia growth and halo of inhibition were reduced in about 70.5% (Table 4). These data support the bioassay results seen in Figure 2 and Table 3 and provide reliability to oil bioactivity. Inhibition assays by SOAD has been reported as a reliable test, in literature. Oxalic acid is naturally produced by the pathogen during parasitism of the host plant (Deacon, 1997; Kucey et al., 1989). This component combines with calcium, favoring the action of pectinolytic enzymes responsible to plant degradation (Deacon, 1997). According to Almeida et al. (2001), the production of oxalic acid may be one of the major factors contributing to wide host range of *S. rolfsii* and is associated with fast fungus development. The progressive accumulation of oxalic acid by fungus leads to a reduction in pH- growth medium, benefiting the formation of sclerotia (Rollins and Dickman, 2001; Maxwell and Lumsden, 1970).

Validation of control *S. rolfsii* with *C. martinii*

In order to confirm the results obtained in bioassays with *C. martini,* a validation assays was carried out in greenhouse. Plant of negative control (seed treated only with water, oil-free) showed characteristic symptoms of white mold, with DSI of 47% (Table 5). No statistical difference was found among oil treatments, whose DSI average was 21.42%, meaning a reduction in 55%, compared to control treatment. Based on these results we suggest that white mold can be controlled through direct seed treatment in a single dose of *C. martini* oil, minimizing others additional costs of application.

Table 3. Sclerotia number of *S. rolfsii* grown in PDA + *C. martinii* oil.

Treatment	Concentration (ppm)								
	0	300	400	500	600	700	800	900	1000
Accession 3	-	0.11b	0.10b	0.04b	0.50b	0.02b	0.02b	0.01bbb	0.02b
Control	113.5a								
Coefficient of variation (%): 2.82									
General average: 4.24									
Standart error: 0.51									
Treatment square mean: 35.32; Freedom degree: 8									
F test: 23.51									

Means with the same letters do not differ statistically by Tukey test ($p < 0.05$). Original data transformed by $\sqrt{(x + 0.5)}$ for statistical analysis.

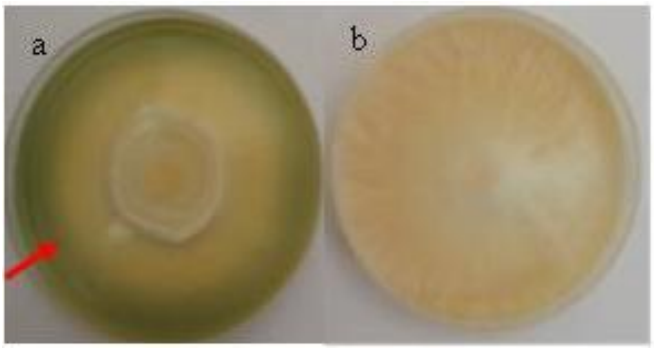

Figure 3. Inhibition of the suppression of oxalic acid diffusion released by *S. rolfsii* in (PDA. **a.** Halo of inhibition with *C. martinii* at 400 ppm (arrow). **b.** Control.

Table 4. Inhibition of the suppression of oxalic acid diffusion in *S. rolfsii* in PDA+ *C. martinii* oil.

Treatment	Mycelia growth (mm)	Halo of inhibition (mm)	Reduction (%)
PDA+ *C. martinii* oil.	26.26a	35.78a	70.5
Control (PDA)	88.75b	1.25b	1.4
Coefficient of variation (%)	4.15	2.64	
General average:	7.28		
Standart error:	1.24		
Treatment square mean:	18.43	Freedom degree: 1	
F test:	201.52		

No statistical differences were found to agronomical traits in fungicide and oil treatments. The control of disease in both treatments allowed gains of 57, 54, and 40% to number of pods, pod weight, and harvest index, respectively. Figure 4 shows a detail of pod production in control and ST treatment. The importance of this result lies in the economic and environmental aspects since *C. martini* oil is cheaper than synthetic fungicides and does not promote environmental damage.

Several studies in literature have highlighted the potential of vegetal essential oils to control plant pathogens. In this study, we confirmed the viability of *C. martinii* oil to control *S. rolfsii*, based on *in vitro* and *in vivo* assays. The trails carried out herein addressed pathogenicity, biochemical (by SOAD) and agronomical assays in order to confirm the effectiveness of *C. martinii* oils against white mold disease. Considering the complexity to control *S. rolfsii* in field, information

Table 5. Disease severity index (DSI) and agronomical traits of BR 1treated with *C. martinii* oil at 400 ppm.

Treatment	DSI (%)	Mature pods/plant		Harvest Index (%)
		Number	Weight (g)	
NC	47.17a	7b	6.7 b	26.53b
PC	13.40c	10a	9.6a	37.93a
ST	21.01b	11a	10.5a	36.91a
ST/11	20.73b	10a	9.5a	35.06a
ST/9	20.80b	10a	9.6a	38.20a
ST/6	21.50b	10a	9.6a	36.84a
ST/3	22.22b	12a	11.4a	37.75a
Coefficient of variation (%)	9.45	12.61	10.78	17.32
General average:	28.12	10.38	10.05	38.46
Standart error:	0.07	0.40	3.51	9.09
Treatment square mean:	345.26	7.49	62.68	172.42
Freedom degree: 6				
F test:	48.86*	4.37*	5.34*	3.89*

Means with the same letters do not differ statistically by Tukey test ($p < 0.05$). NC: negative control (seeds treated with water, oil-free); PC: seeds previously treated with commercial fungicide (positive control, oil-free); ST: seeds previously treated with essential oil; ST/11- seeds previously treated with essential oil + 11 weekly applications of oil at same concentration; ST/9- ibid + 9 applications of decennial oil at the same concentration; ST/6- ibid + 6 biweekly applications of oil at the same concentration; and ST/3- ibid + 3 monthly applications of oil at the same concentration.

Figure 4. Pod production in peanut inoculated with *S. rolfsii*. A. control (seeds treated with water); B. seeds previously treated with essential oil at 400 ppm (ST treatment).

contained in this study provides an alternative to minimize the losses in peanut production and damaging to the environment.

Conflict of interests

The authors did not declare any conflict of interest.

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Quality of honey sold in the state of Alagoas, Brasil

Thaís Patrícia Alves, Tania Marta Carvalho dos Santos*, Cícero Cerqueira Cavalcanti Neto, Roger Nicolas Beelen, Sybelle Georgia Mesquita da Silva and Yamina Coentro Montaldo

Universidade Federal de Alagoas. Centro de Ciências Agrárias, BR 104 Norte, Km 85, Rio Largo, Alagoas, Brasil.

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The present study aimed to determine the quality of honey marketed in the State of Alagoas, Brazil. Fifteen samples of *Apis mellifera* L. honey sold in supermarkets, free trade, and cooperative located in the State of Alagoas were acquired. Microbiological and physical-chemical analyzes were carried out to establish a standard microbiology condition and check for possible tampering. The physico-chemical analyzes showed that all the samples studied presented acid pH values ranging between 2.3 and 4.4. For diastase activity and reaction, Lugol which are indicative of the presence of starch and dextrin, and reaction Fiehe, which is a qualitative indicator of HMF, all samples were negative for at least the parameters. As the microbiological standard, 26.6% of all samples showed high standard count mesophilic aerobic bacteria, 20% had counts of molds and yeasts above the quality standards established by Brazilian law. For enumeration of coliforms at 35 and 45°C, it was found that most samples were contaminated (86.7%). It is the presence of sporulated bacteria in 13.3% of the samples, which were 15.26 and 84.64% genus *Clostridium* of the genus *Bacillus.*

Key words: Apiculture products, contamination, physico-chemistry, microbiology, *Clostridium botulinum.*

INTRODUCTION

Honey is a complex mixture of sugars (35% glucose, 40% fructose, and 5% sucrose) and highly concentrated organic acids, enzymes, vitamins, flavonoids, mineral and a wide variety of organic compounds that contribute to its characteristics sensory and nutritional (Serrano, 1994). Its composition depends on the nectar of the components of the production plant which it gives the product its specific characteristics.

Honey is an acid food, with low humidity and water activity. Its viscosity is high due to high concentrations of sugars, and osmotic pressure. These conditions make honey slightly favorable substrate for microbial development. However, it may be caused by the bee microflora itself, lack of hygiene in the extraction and processing, including pollen, floral nectar, dust, dirt and the body itself and bee digestive tract, as well as fungi and some bacteria (Snowdon and Cliver, 1996; Bogdanov, 2006; Rissato et al., 2007; Rial-Otero et al, 2007; Kujawski and Namiesnik, 2008).

Another factor rarely considered is the length of the production cycle. The time of flowering station can interfere with the microbiological quality of honey since in

*Corresponding author. E-mail: tesfalem2002@gmail.com.

low food availability, bees can forage in fungal colonies (Snowdon, 1999) or even feces and other sources of organic matter (Nogueira Neto, 1997).

The osmophilics microorganisms comprise those able to grow and multiply in honey (Ward and Trueman, 2001); other microbial groups which can be found in honey are spore-forming bacteria. These microorganisms can be directly related to the deterioration of the product, production of enzymes, toxins, metabolic conversion of food, the production of growth factors (vitamins and amino acids) and inhibition factors of competing microorganisms (Silva et al., 2008). Usually acidic, high water activity and high humidity are the main factors responsible for the development of these microorganisms (Bogdanov, 2009). The microbiological analysis to determine which and how many microorganisms are present are of fundamental importance to know the hygiene conditions in which food was prepared, the risks that food can offer the consumer health and life span required. This analysis is necessary also to verify that standards and microbiological specifications for foods, domestic or international, are being met adequately.

Honey is subject to variations in its aroma, taste, color, viscosity and medicinal properties. However, these features can also be modified by tampering the generation by unreliable sources who misuse the product, adding in composition lower commercial substances and nutritional value (Ribeiro et al., 2009). Tampering is generally carried out with the addition of other carbohydrates, particularly sugars such as commercial glucose solution or sucrose syrup and invert sucrose solution, from cane or corn (Rossi et al., 1999).

These changes are detected by domel physical-chemical analysis, as in the case of qualitative analysis of hydroxymethylfurfural (Reaçãode Fiehe) which, when in high concentration shows the heating of honey, or addition of sugar syrups or artificial feeding of bees honey. Bodganov et al. (1997) reported that honey damage caused by heating can be evidenced by determining the HMF content and activity of the diastase, since these parameters together are used as indicators for intensive heating (Ramirez et al., 2000). According to Wiese (2000), the lugol test reaction indicates the adulteration of starch and dextrin which does not occur in pure honey. Another analysis is pH, which when below or above the level permitted, can favor the growth of bacteria, which can spoil the honey and affect the quality, as well as the acidity analysis when at high level.

The objective of this study was to evaluate the *Apis mellifera* bee honey quality marketed in the state of Alagoas- Brazil through analysis of microbiological and physical-chemical parameters.

MATERIALS AND METHODS

The experiment was conducted in the microbiology laboratory at the Academic Unit Centre for Agricultural Sciences (CECA-UFAL), located on Rio Largo district, Zona da Mata Alagoas (9 27' latitude 54.8" S and longitude 35° 49' 59 7" W), from January to May 2013. The city is situated at an altitude of 127 m, with average maximum temperatures of 29°C and minimum of 21°C and average annual rainfall of 1,268 mm.

Honey samples

The samples were acquired at collection points such as supermarkets, grocery stores, and cooperative located in the State of Alagoas. From November to December 2012, we obtained 15 samples of honey from *A. mellifera* L., where five were acquired in own commercial packaging of independent apiaries produced in this state, settled (had some inspection seal) or not; bee different regions of the State of Alagoas (MM1, MM2, MM3, MM4 andd MM5) and another 10 provided by coopmel (Mel Cooperative State MC6, MC7, MC8, MC9, MC10, MC11, MC12, MC13, MC14 and MC15). All samples were taken to the Academic Unit of Microbiology Laboratory Centre of Agricultural Sciences, Federal University of Alagoas, where they were examined.

Processing of samples

Twenty five grams of each sample (were aseptically collected and added with 225 mL of 0.1% sterile peptone water (SPW), that had 1:10 dilution, were homogenized in shaker orbital at 2,000 rpm for 30 min. The total mesophilic aerobic and psychrotrophic bacteria counts were carried out in pour plate using plate count agar (PCA) followed by incubation at 35°C for 48 h for mesophilic bacteria.

Coliforms at 35 and 45°C were counted through the most probable number (MPN), with three sets of three tubes. Lauryl sulfate tryptose broth (LST) was used as a presumptive medium and incubated at 35°C for 24-48 h. After reading, the positive tubes were transferred to brilliant green bile broth (2%, GB) and EC broth. Then was incubated at 35°C for 24-48 h; for confirmation of total coliforms and EC broth tubes, they were incubated in a water bath at 45°C for 24 h for confirmation of thermotolerant coliforms.

The homogenate used for microbiological characterization was subsequently used for the isolation of bacteria. Isolation of *Clostridium* was performed by seeding 1 ml each decimal serial dilution in triplicate in 10 mL of Cooked Meat Medium (CMM); the tubes were immediately moved to a water bath at 65°C for 30 min in order to inactivate the microorganisms spore. The samples were incubated at 35°C for seven days.

After the incubation period, the cultures were observed for turbidity, gas production, and digestion of meat particles in broth. Cultures with insignificant growth were reincubated in the oven at the same temperature previously used for three days, completing a maximum period of ten days. Cultures still without growth were discarded because they were considered negative.

The positive samples were subjected to Gram's method for detection of Gram-positive bacilli sporulated or not. Positive cultures were seeded to Petri plates containing Anaerobic Egg Yolk Agar (AEY) and incubated anaerobically in Colorina pot, at 35°C for seven days. Typical obtained colonies were re-isolated in plate in duplicate in medium containing AEY and each incubated aerobically and anaerobically at Colorina pot; both at 35°C for 48 h. Later blades were made for the plates for staining by the gram method to detect Gram-positive bacilli.

The isolation of yeasts and molds was carried out using 0.1 mL of seeding on the surface of each agar dilution dicloran Rose Bengal Chloramphenicol, followed by incubation at 25°C for five days. The colony forming units were calculated using the following formula:

$$CFU\ g = X.DF/ V$$

Where, X = average of each dilution, DF = dilution Factor and V =

Table 1. Microbiological parameters of honey of *Apis mellifera* obtained from independent beekeepers and cooperative in the state of Alagoas-Brazil.

Sample	Aerobic mesophilic bacteria	Molds and yeasts	Coliforms	
			35°C	45°C
		CFU.g^{-1}	MPN.g^{-1}	
MM1	1.5x10^{7}	-	0.20	0.15
MM2	-	-	0.16	0.09
MM3	-	-	>24.00	0.53
MM4	-	-	>24.00	0.44
MM5	-	2.2x10^{7}	<0.03	<0.03
MC6	-	-	0.04	0.04
MC7	-	-	0.09	0.03
MC8	-	3.4x10^{7}	0.04	0.07
MC9	-	-	<0.03	<0.03
MC10	7.4x10^{5}	2.5x10^{7}	>24.00	0.44
MC11	-	-	0.19	0.12
MC12	-	-	>24.00	0.75
MC13	4.2x10^{4}	-	0.19	0.03
MC14	1.7x10^{6}	-	>24.00	<0.03
MC15	-	-	0.03	0.06

volume dilution added to the Petri dish

Determination of pH, qualitative test HMF (Fiehe reaction); lugol reaction and determination of diastase activity were performed according to the methods proposed in the standards of the Institute (Adolfo Lutz, 2008). All analyses were done in triplicate and the mean values were used for the statistical evaluation.

The results were submitted to descriptive statistics (mean, standard deviation and coefficient of variation). For statistical analysis, logarithmic transformation (log10) was used for mesophilic microorganisms count, MPN of coliforms, molds and yeast to in order to normalize the distribution frequency.

RESULTS AND DISCUSSION

Table 1 shows the results of the microbiological analyzes of the samples. The presence of the mesophilic aerobic bacteria on four samples were detected which corresponds to 26.6%. The maximum and minimum values MM1 and MC13 samples were respectively 7.4x10^{5} and 4.2x10^{4} CFU.g^{-1}. Presence of yeasts and molds was observed in samples MC5, MM8 and MM10 (20% of the samples); the values obtained were 2.2x10^{7}, 3.4x10^{7} and 2.5x10^{7} CFUg^{-1} respectively. With respect to coliforms at 35°C and coliforms at 45°C, it was observed that only 2 (13.3%) samples had lower results than 3.0 MPNg^{-1}, that is absence in 86.7% of samples; a high rate of contamination was detected in four of them and the presence of coliforms at 35°C was observed higher than the level 24.0 MPNg^{-1}.

Chemical and physical properties of honey can inhibit or destroy the microorganisms. Several authors report showed a strong antibacterial activity, including human and animal pathogens (Iurlina and Fritz, 2005; Kačaniová et al., 2009; Adenakan et al., 2010).

However, the honey production and processing involves different steps through which some microorganisms can survive or even multiply. Primary sources of microbial contamination probably include the pollen, the digestive tracts of honeybees, dust, air, earth and nectar - sources that are very difficult to control. The same secondary (post-harvest) sources that influence other food products are also sources of contamination for honey. These include air, food handlers, cross-contamination, equipment and buildings. Secondary sources of contamination are controlled by good manufacturing practices (Kačániová, 2004; Olaitan et al., 2007).

The Brazilian legislation (Brazil, 2000) does not set values for mesophilic aerobic bacteria in honey but establishes only that you follow good hygiene practices in handling and processing of this product because entire microbial load in honey can indicate the possible presence of pathogens. Therefore, the default score has been used as an indicator of hygienic quality of food, including the cleaning, disinfection and control of environmental health during processing, transport and storage, and providing also of idea about its useful shelf life.

The results were superior to those obtained by Malika et al. (2005) and Schlabitz et al. (2010) and lower than those presented by Melo (2013). According to Snowdon and Cliver (1996) variation in the number of bacteria seems to depend on the type of sample, the age and the honey harvest time. These vegetative forms can be made by secondary contamination which would also explain the high counts sometimes found in honey.

The results obtained for standard counting of molds

and yeasts showed that 20% of samples had values above the maximum established by the Brazilian technical standards for food, RDC 012 (Brazil, 2001), being considered unfit for direct human consumption.

Snowdon and Cliver (1996) found that yeast is one of the most important microorganisms that interfere with the quality of honey. Typically this yeast presence in the samples, can be detected in high concentrations; they survive under acidic conditions and are not inhibited by sucrose. These osmophilic yeasts (tolerant sugar) represent a problem in honey industry because they have the ability to grow at low water activity.

The contamination in honey may occur naturally, where the fungi are brought to the hive by bees or by the absence of the use of good apicultural practices during handling of the hives; it is worth emphasizing the importance of continuous monitoring throughout the honey processing, to ensure the marketing of a reliable food.

The presence of yeasts and molds is generally accepted for all honey, however the biggest problem is related to fermentation of the product, resulting in the hydrolysis of sugars to produce alcohol and carbon dioxide, changing the taste and the flavour of honey (White Jr, 1978).

In fresh honey, the number of yeasts and molds is generally low, but under certain conditions these organisms are able to multiply in honey during storage, especially in honeys with high moisture content and water activity (Martins et al., 2003; Iurlina and Fritz, 2009; Kačaniová et al., 2009; Carvalho et al., 2010; Różańska and Osek, 2012). Jimenez et al. (1994) observed a significant increase in the number of yeasts and molds with storage time.

Other work related to the quantification of micro-organisms in honey found similar results. In Cameroon honey samples, Tchoumboue et al. (2007) found the presence of contamination by microorganisms in more than 73.4% of the samples, attributing this contamination to post-harvest processing or tampering of the product, since their witness honey sample did not show these levels of contamination. Finola et al. (2007) determined that lower count of 1.0x10 $CFUg^{-1}$ in molds and yeasts in all samples.

The results observed for coliforms at 35°C, suggest a failure to follow good practices of manipulation of honey and that the presence of these microorganisms also constitutes an indicator of the possible presence of other pathogenic microorganisms that are more difficult to detect. The presence of enterobacteria in total honey originates from fecal contamination which is evidence of poor condition of extraction and processing and their own marketing.

The results coincide with those obtained by other authors. Gomes et al. (2010) isolated *Salmonella* spp., *Coliforms* and *E. coli* in Portugal at a 34% rate; Kokubo et al. (1984) analyzed 70 samples of honey and isolated coliforms at a rate of 95.7%. Dumen et al. (2013) studied the honey produced in Istanbul and verified the presence of coliforms in 18% of 80 samples.

Table 2. Gram positive confirmation in culture medium AEY.

Sample	Anaerobic organism	Aerobic organism
MC4	+	+
MM9	+	+

The major quantitative indicators of microorganisms can be related to the collection period of pollen by bees. According to Barth (2004) when there is shortage of flowers, bees can forage in the most diverse substrates, from fungal colonies through soil, clay and even matter organic fecal origin. Based on this, it is desirable that areas close to breeding sites are free from other ranchers activities such as the creation of other animals. Matos et al. (2011) found that honey samples collected from hives that had potential contamination sources in the environment such as cattle dung, showed high counts of these microorganisms.

For the detection of *Clostridium* sulfite reducers in the samples, analyzes were performed by means of cooked meat; after the incubation period, 13 samples were discarded by negative results, they were: MC1, MC2, MC3, MC5, MM6, MM7, MM8 , MC10, MC11, MC12, MC13, MC14, MC15; the cultures in which they observed turbidity, gas production, digestion of meat particles in the broth represented a total of 13.3% of the samples and these were subjected to Gram's method for detection of Gram-positive bacilli sporulated or not.

The two positive samples were stained by the Gram method, and the presence of Gram-positive bacilli were detected and then were passed to the Petri dishes containing the AEY, incubated aerobically and anaerobically and submitted again to the Gram stain for confirmation of Gram-positive bacilli. The results are shown in Table 2.

The results of this study demonstrate the presence of sporulated bacteria in 13.3% of the samples identified by smear slide and stained by the Gram method, both under aerobic and anaerobic conditions. Biochemical tests showed that 15.26% were genus *Clostridium* and 84.64% of the genus *Bacillus*.

Although honey is a hostile environment for the growth of food-borne pathogenic bacteria, spores and vegetative latent forms may be present due to primary and secondary contamination. Spore-forming bacteria such as *Bacillus cereus* and *Clostridium* spp. are regularly found in honey. Pucciarelli et al. (2014) found the incidence of *Clostridium* and *Bacillus* (42.85 and 39% respectively) in yateí honey, Argentina. Ragazani et al. (2008) studying honey marketed in several Brazilian states found 39% sulfite-reducing bacteria, and 11% were *Clostridium* genus and 28% of the genus *Bacillus*.

Table 3. pH, diastatic activity, lugol reaction and qualitative analysis of hydroxymethylfurfural (Fiehe reaction) in honey bees *Apis mellifera* L. marketed in Alagoas-Brazil.

Sample	pH	Diastase activity	Lugol's iodine reaction	Fiehe reaction
MM1	2.4	-	+	-
MM2	2.7	-	-	+
MM3	3.0	-	-	+
MM4	2.5	-	+	+
MM5	2.3	-	+	-
MC6	2.8	-	-	-
MC7	3.5	+	-	+
MC8	3.1	-	-	+
MC9	3.6	-	-	+
MC10	4.4	+	-	+
MC11	3.3	-	-	-
MC12	3.6	-	-	+
MC13	3.0	-	-	+
MC14	3.0	-	-	+
MC15	3.4	-	-	+

The presence of bacteria of the genus *Bacillus* spp. honey would be expected, since there is a symbiotic relationship between these microorganisms and insects including bees (Nicholson, 2002). *C. botulinum* is a bacterium of the bacterial type, straight or semi-curved, gram-positive spore, mobile, strictly anaerobic and has sulfite-reducing activity that is common in soil, air and environmental waters and can be found in various foods. This bacterium produces toxins that cause digestive and neurological disorders in the patient; the disease known as botulism is a very serious disease.

The incidence of *C. botulinum* spores in honey has been estimated in several studies. Sugiyama et al. (1978), using the dialysis method of 241 samples of honey in USA, reported the presence of *C. botulinum* spores in samples originating from 18 States: California, Florida, Iowa, Michigan, Minnesota, Nebraska, Tennessee, Texas and Washington etc. In experiment conducted by Midura et al. (1979), *C. botulinum* were isolated from nine samples from 90 honey samples analyzed. Among these, six samples were provided to infants, who developed the disease.

Küplülü et al. (2006) isolated *C. botulinum* from 12.5% of the retail market honey samples in Ankara, Turkey. Ragazani et al. (2008) isolated bacteria from 11% of the samples; Schocken-Hurrino et al. (1999) detected *C. botulinum* in 7% of Brazilian honey samples.

The evidence of tampering were carried out according to the Analytical Standards Institute Adolfo Lutz. All analyzes were performed in triplicate. The results of the physical-chemical analysis are presented in Table 3.

All samples had pH values ranging between 2.3 and 4.4; for enzymatic activity only two honey (13.3%) MC7 and MC10 showed positive result. Three samples (20%) showed a positive reaction to lugol. Regarding the Fiehe reaction, 73.3% of samples (11) were salmon-colored red cherry, that is positive reaction.

There is no national or international rules setting limits for pH (Silva et al., 2004) but it is a very important parameter for obtaining and honey storage for its influence on the development of microorganisms and enzymes. It also affects the physical properties of the product such as a texture, stability and resistance.

Variations in pH observed, according to Crane (1990) are probably due to peculiarities of the composition Floristics collection areas, since the pH of the honey can be influenced by the pH of nectar. In addition, differences in soil composition, or the association of plant species for final composition of honey, can also influence the pH of this product.

Substances present in the jaw bees are added during transport to the hive which can change this factor. All samples showed acid pH; the acids added by bees contribute to the taste of the honey and stability against microbial growth, and the main gluconic acid resulting from oxidation of glucose by glucose oxidase (Bogdanov et al., 2004).

The most important enzyme in honey is invertase, also known as sucrase, whose function is to convert nectar honey, since it acts by hydrolyzing sucrose and generating final products, glucose and fructose (White, 1975).

According to Huidobro and Simal (1984a) there are three very important enzymes for honey: amylase, invertase and glucose oxidase. The diastase activity in honey, usually quantified by α-amylase, is a quality factor that can be changed during processing and storage of honey, so it is used as heating and freshness indicator (Bogdanov et al., 2006).

The diastase activity varies with the botanical origin of

honey; many countries require minimum amounts of diastase or amylase activity, which is easily degraded by aging and the action of heat, disappearing half its content in 17 months at room temperature. However, when interpreting the results of diastase activity, one must consider that some monofloral honeys such as the citrus have a natural low activity (Huidobro and Simal, 1984a), implying an analysis that has limited power as deterioration indicator (Bogdanov et al., 1997).

Lugol reaction yielded positive results indicating the presence of starch and dextrin in three (20%) samples. Honey is formed carbohydrates composed of mono- and oligosaccharides obtained from flower nectar, which does not have in its composition polysaccharides such as starch. The reaction with Lugol's shows the presence of starch (large molecule formed by the union of several hundred glucose molecules / natural energy reserve of the plants) and dextrin (polysaccharide class of low molecular weight) in honey. The positive result is indicative of adulteration of the product with starch and dextrin.

Regarding the reaction Fiehe, 73.3% of the samples had salmon color red cherry, that is positive reaction to the test, being at odds with Brazilian legislation (Brazil, 2000). The hydroxymethylfurfural (HMF) is not a normal component of honey; it is a cyclic aldehyde formed at room temperature by fructose dehydration in acid medium (pH 3.9), a process that is accelerated by heating or storage at elevated temperatures (Huidobro and Simal, 1984b).

The content of HMF, is directly related to the heat that has undergone honey and the degree of aging (Bosch and Serra, 1986). Its presence causes the browning interactions with amino compounds and sugars, undergoing polymerization and rearrangement in the presence or in the absence of oxygen. The results indicate that these samples may have been subjected to overheating conditions, high temperature or stored with addition of sugar syrup or corresponds to an old honey. The HMF concentration is also associated with the existing enzyme activity, so that those honeys with low index diastases possibly have high numbers of hydroxymethylfurfural which would be indicative of improper storage.

Conclusion

At the end of this study, it was observed that none of the samples showed all microbiological and physico-chemical parameters within acceptable limits. With tamper analysis, it was observed that there is a need to identify factors that result in overheating of these samples so that preventative measures can be taken, since in these cases important properties of honey may be lost. The quality of honey can be affected by management during harvest, thus the beekeeper must perform the appropriate procedures from the time of withdrawal of honey from hives to transportation of the extraction unit, in order to interfere as little as possible with the hygienic sanitary quality.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Response surface optimization of xylanase production by indigenous thermoalkalophillic *Bacillus* sp.

Shanthi V.* and Roymon M. G.

Department of Microbiology, St. Thomas College, Bhilai, Chhattisgarh- 490006; India.

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Xylanases are an important class of hydrolytic enzymes with a wide range of industrially important applications especially in paper and pulp industry. The present study aimed to take the advantage of statistical approach of optimization to investigate the interactive effects of prominent process factors involved in xylanase production. A novel bacterial isolate *Bacillus* sp. MCC 2727 was isolated from soil possessing xylanase producing ability at alkaline pH (9.2) and optimum temperature of 50°C. Using the conventional one-factor-at-a-time method, low cost agricultural waste; wheat bran, combination of peptone and yeast extract served as best carbon and nitrogen sources, respectively. $MgSO_4$ as metal salt and xylan as additive increased the xylanase productivity. Central composite design and response surface methodology were used to optimize these significant process parameters and for evaluation of interactive factors. Maximum xylanase activity of 205.3 IU/ml was obtained with 5% wheat bran, 1% each of yeast extract, peptone, xylan and $MgSO_4$ which was in consensus with the predicted value (207.2 IU/ml) which proved the validity and the accuracy of the statistical approach of optimization.

Key words: Xylanase, response surface methodology, central composite design, optimization.

INTRODUCTION

Hemicelluloses are considered as the second most abundant polysaccharides in nature after cellulose. The most common hemicelluloses found in plants and trees are xylan. Xylan is also found in solid agricultural and agro industrial residues (Collins et al., 2005). These solid wastes can be potentially used to produce various industrially useful products like biofuels, animal feed, enzymes etc. (Abo-State et al., 2013). Xylanases are the most important xylan degrading enzymes. They have created a niche for themselves in the field of enzyme technology for the good reason that they have immense biotechnological applications. Most of the industrial applications including paper and pulp require that xylanases have a high temperature and pH optima. Although efficient producers; fungal xylanases are associated with a plethora of problems. Bacteria are more appealing compared to fungi as they are very easy to cultivate. Also bacterial xylanases have a high temperature and pH optima (Subramaniyan and Prema, 2002).

The industrial applicability of enzymes is determined by its production costs. The process economy mainly relies on the optimization of the media components leading to higher yields (Kanagasabai et al., 2013). The

*Corresponding author. E-mail: shanti_162@rediffmail.com. Tel: 9406103610.

conventional method of optimization using one- variable-at-a-time is both tedious and time consuming. One of the popular methods for optimization of different parameters affecting productivity of enzymes is response surface methodology (RSM). In recent years, RSM has found significant importance in various biochemical and biotechnological processes (Bas and Boyaci, 2007). The inability of the conventional method to explain the extent of effect of variables on the response and also the interactive effects of the process parameters can be overcome by a more satisfactory method of statistical optimization. Central Composite Design and Response Surface Methodology are efficient strategies of optimization of medium components.

MATERIALS AND METHODS

Microorganism

Several soil samples collected from local Bhilai region of Durg District, Chhattisgarh; India was screened for potent xylanase producing bacterial strains. The preliminary screening was performed on xylan agar medium (pH 9.2) and incubated at 50°C for selection of alkalophillic thermostable isolates. The secondary screening of the isolates from the preliminary screening procedures was performed by Congo red plate assay for the detection of clear zone around the colonies. Isolate showing maximum zone of xylan hydrolysis was selected and sent to National Center for Cell Science (NCCS), Pune; Maharashtra, India for identification on the basis of phenotypic and molecular characterization. The pure culture was maintained and stored on nutrient agar slants at 4°C for further use.

Xylanase production by submerged fermentation

20 ml of liquid basal medium containing 0.5% Birchwood xylan, 0.5% Peptone, 0.5% yeast extract, 0.2% K_2HPO_4 and 0.01% $MgSO_4.7H_2O$ in 100 ml Erlenmeyer flask was sterilized by autoclaving at 121ºC for 20 min and cooled to room temperature. The pH of the medium was adjusted to 9.2 by adding sterile 10% Na_2CO_3 solution after sterilization. The flask was inoculated with 1% v/v of 18 h old fresh inoculum and incubated at 50°C for 48 h on a rotary shaker at 150 rpm. After the desired interval, the contents were subjected to enzyme extraction.

Enzyme extraction and xylanase assay

Crude enzyme was extracted from the fermentation broth by centrifugation at 10,000 *g* for 10 min at 4°C (REMI, Cooling centrifuge; C-24BL, India). The supernatant obtained was used as a source of crude xylanase enzyme. The quantitative estimation of xylanase activity was done with some modifications according to the procedure of Sharma et al. (2013). A reaction mixture was prepared containing 0.5 ml supernatant and 0.5 ml of 1% Birchwood xylan (HiMedia, India) solution prepared in 50 mM Glycine-NaOH buffer (pH 9.2). The reaction was terminated by adding 3 ml DNS reagent after incubating at 55°C for 10 min. The mixture was kept in boiling water for 5 min and cooled. The amount of reducing sugar (xylose equivalents) liberated was determined according to Miller (1959). One unit (IU) of xylanase activity is defined as the amount of enzyme required to release 1 µmol of xylose per minute under the specified assay conditions. The results presented are the mean of three values obtained from experiments performed in triplicates.

Optimization of xylanase production

The optimization studies included both physico-chemical parameters and nutritional parameters. The different important parameters governing the production of xylanase were optimized by the conventional one-factor-at-a-time method (Results not shown).

The best carbon source was selected from about twelve different carbon sources which included both simple and complex forms of carbon. Nine different Nitrogen sources including both organic and inorganic forms of nitrogen were used for optimization of best nitrogen source. Optimization of additives and metal salts on production of xylanase enzyme was also optimized. Using the conventional method of optimization, the important factors which affected xylanase production were wheat bran (best carbon source), xylan (additive), $MgSO_4$ (best metal salt), peptone and yeast extract (best carbon source).

Response surface methodology (RSM)

A statistical method, Central Composite Design (CCD) was adopted to optimize five different variables: carbon source (wheat bran), nitrogen source (peptone and yeast extract) $MgSO_4$ and additive (xylan). Each variable was taken at five coded levels (-α, -1, 0, +1, +α). The variables and their coded values are shown in Figure 1. The optimization using RSM by CCD is an efficient statistical method for optimization of process variables and also helps to evaluate the interaction between the dependent variables. The statistical software package Design- Expert (version 9.0.3.1, Stat-Ease, Minneapolis; USA) was used to design the experiment and calculate the coefficients. The central coded values of all the variables were taken as '0'. The statistical significance of the linear and quadratic effects generated by the model equation was tested by applying F-test. Analysis of Variance (ANOVA) was used to estimate the various statistical parameters.

RESULTS AND DISCUSSION

Isolates from different soil samples were screened for their xylanolytic property on xylan agar medium in the preliminary screening process. Ten bacterial isolates showed good growth on the medium when grown at specified conditions indicating alkalophillic and thermostable nature of which one of the isolate produced maximum clear zone of xylan hydrolysis in the secondary screening by Congo red method. This selected isolate was motile, catalase positive, Gram positive thin rods with sub terminal ellipsoidal spores. The identification reports from NCCS, Pune; Maharashtra, India confirmed the strain belonged to Bacillus sp. and was given the accession number MCC 2727 (Table 1). The identification reports from NCCS, Pune; Maharashtra, India confirmed the strain belonged to Bacillus sp. and was given the accession number MCC 2727.

Optimization using RSM

The effect of five different variables (Wheat bran, Yeast extract, Xylan, $MgSO_4$ and Peptone) on xylanase enzyme production was evaluated by CCD and RSM. The CCD package helps to study interactive effect between the

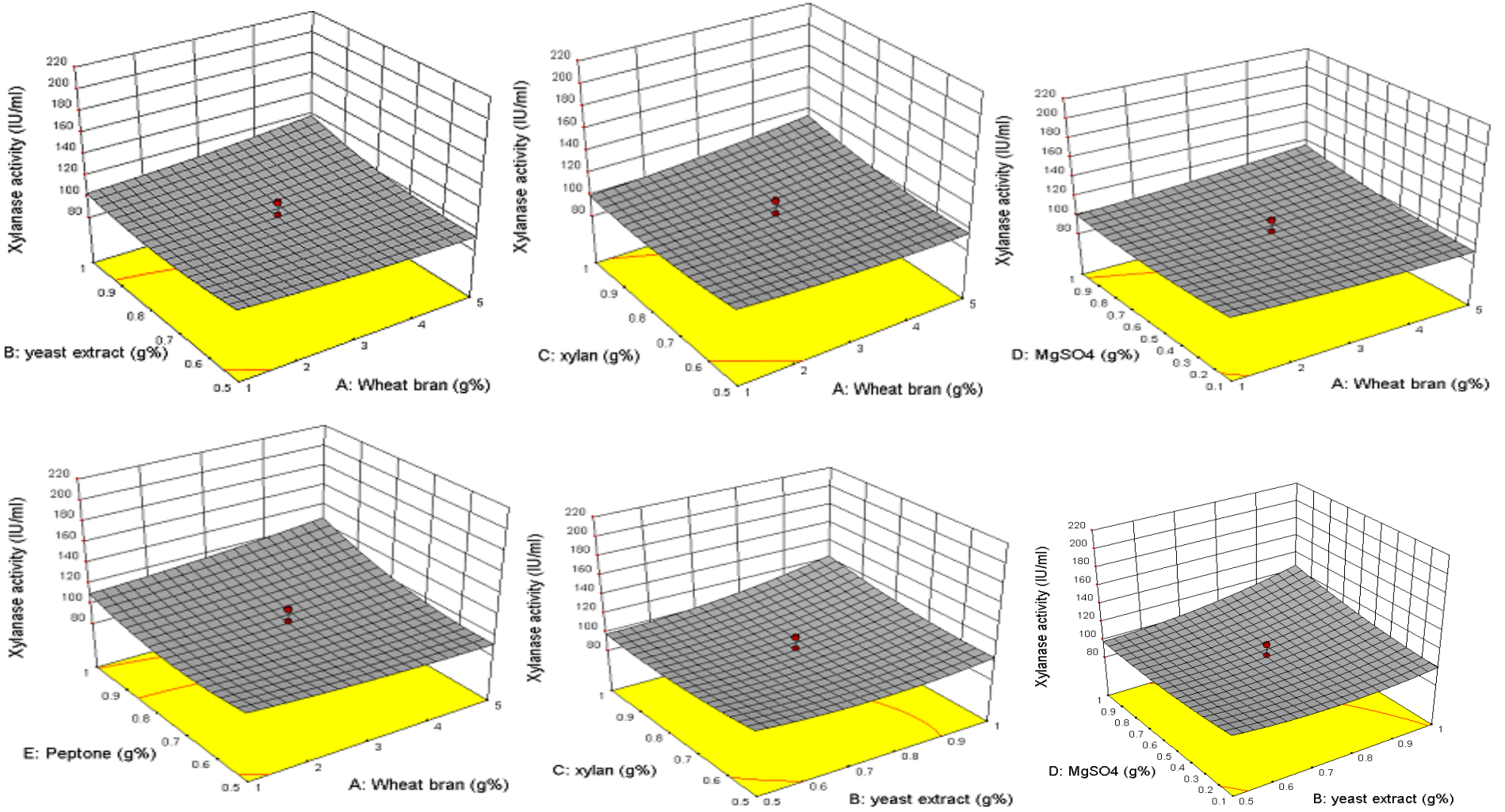

Figure 1. 3-D Response curves of xylanase production from *Bacillus* sp. MCC 2727, showing interactions between various variables.

different variables while the RSM helps to predict and evaluate the optimum variable concentrations aiding in obtaining high enzyme yields (Garai and Kumar, 2013). In the present study, the significance of coefficients of both linear and quadratic terms was tested through the p value. Analysis of variance (ANOVA) results of the CCD model are shown in Figure 2. P values < 0.05 are considered significant and p values < 0.0001 are highly significant (Zambare, 2011). The coefficients of linear model term values B (Yeast extract), C (Xylan), D ($MgSO_4$) and E (xylan) were found to

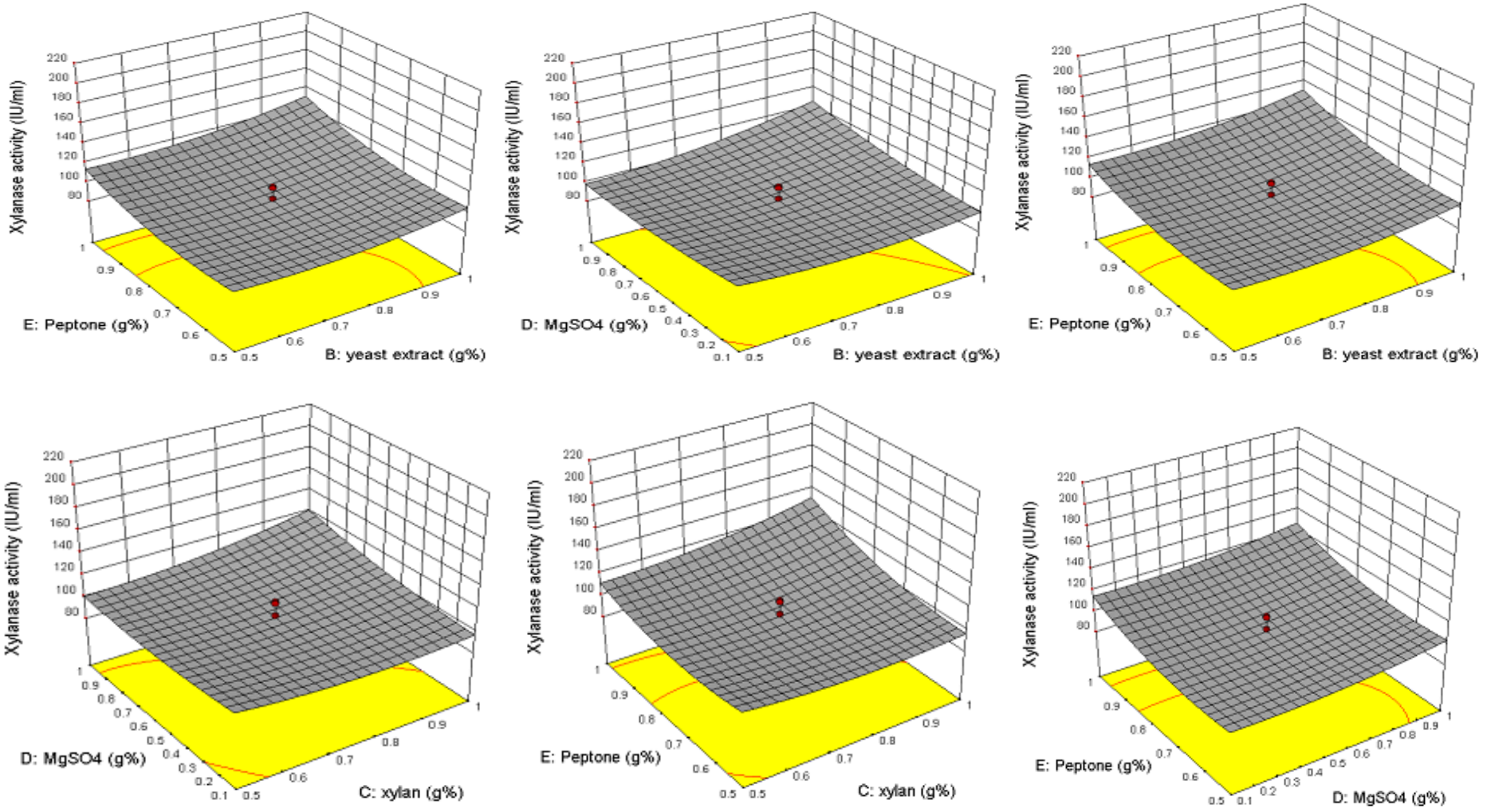

Figure 1. Contd.

significantly affect the productivity of enzyme. This implies that these variables may be acting as limiting medium components indicating that even little change in their concentrations will affect the xylanase production.

The p value of coefficients of quadratic model terms except BE and DE were found to be significant indicating interactive effect between most of the process variables.

The model F value is 32.53 implying the significance of the model. There is only 0.01% chance that F value this large could occur due to

Table 1. Variables and their coded levels for CCD.

Variable (g%)	Code	Coded level of variables				
		-α	-1	0	+1	+α
Wheat bran	A	-1.75	1.0	3.0	5	7.75
Yeast extract	B	0.15	0.5	0.75	1	1.34
Xylan	C	0.15	0.5	0.75	1	1.34
MgSO4	D	-0.52	0.1	0.55	1	1.62
Peptone	E	0.15	0.5	0.75	1	1.34

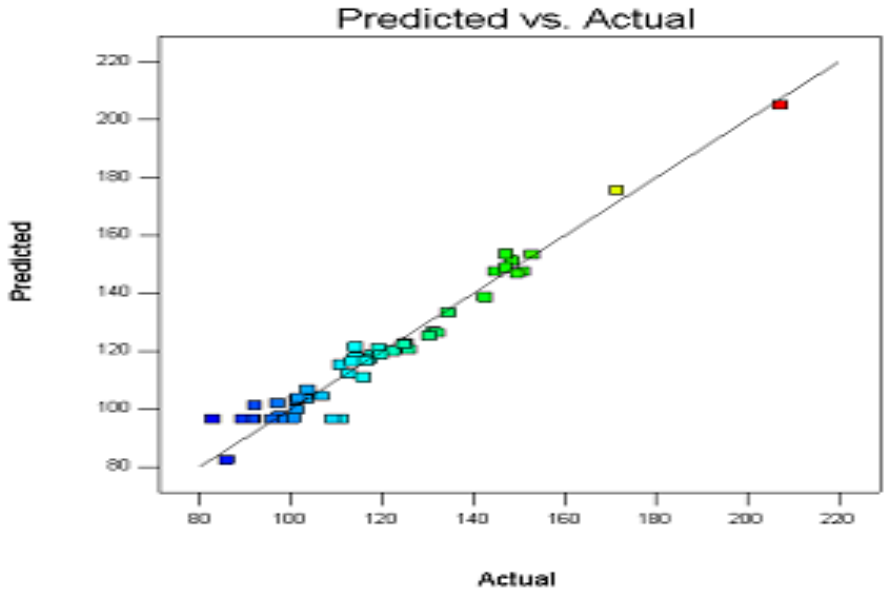

Figure 2. Parity plot of xylanase production showing correlation between predicted and experimental values.

noise.

The coefficient of determination; R^2 and Adjusted R^2 were calculated to check the Goodness of fit of the model. The values of R^2 lie in the range of 0.0-1.0 (Amani et al., 2007). The R^2 value for this model was found to be 0.9573 which is very close to 1.0 implying the accuracy of the model and better response prediction (Table 2).

The second order regression equation showing the relationship between Y (Xylanase Activity) and the five process variables in terms of coded values is given as:

$$Y= 96.3699 + 1.74523A + 6.62775B + 3.54476C + 5.60661D + 11.1619E + 5.82281AB + 5.63906AC + 5.84406AD + 6.69281AE + 6.30531BC + 7.26531BD + 2.10031BE + 6.68656CD + 6.52531CE + 0.357812DE + 2.04623A^2 + 6.1121B^2 + 5.01608C^2 + 4.15871D^2 + 9.2764E^2$$

Higher model R^2 values however always do not indicate model accuracy as inclusion of extra non-significant variables may also lead to their higher values. Adjusted R^2 values are therefore considered which manages the R^2 values according to the number of model variables (Cooman and Bahrin, 2011). The more the number of extra insignificant variables, the decreased will be the adjusted R^2 value. Ideally, for the model to be highly significant and for better response prediction, the value of R^2 should be as close to as possible to Adjusted R^2 value. The R^2 value of 0.9279 indicates that 92.79% of the variability of the response can be explained by this model. The signal to noise ratio is measured by adequate precision value which for this model is 29.441 which is greater than the desirable value of 4.0 indicating adequate signal. Simultaneously lower values of coefficient of variation (CV= 5.38%) indicates high precision and reliability of the design model.

The interactive effect between any two independent variables on xylanase production keeping the remaining variables at their central coded level can be studied from the 3D surface curves and contour plots. Elliptical contour plots indicate significant interaction between the corresponding variables while insignificant interaction by

Table 2. Analysis of variance (ANOVA) for the CCD design model.

Source	Sum of squares	Degree of freedom	Mean of squares	F Value	P Value
Model	26839.16	20	1341.96	32.53	0.0001*
A	131.93	1	131.93	3.20	0.0842
B	1902.64	1	1902.64	46.12	0.0001*
C	544.25	1	544.25	13.19	0.0011*
D	1361.53	1	1361.53	33.01	0.0001*
E	5396.39	1	5396.39	130.82	0.0001*
AB	1084.96	1	1084.96	26.30	0.0001*
AC	1017.57	1	1017.57	24.67	0.0001*
AD	1092.90	1	1092.90	26.49	0.0001*
AE	1433.40	1	1433.40	34.75	0.0001*
BC	1272.22	1	1272.22	30.84	0.0001*
BD	1689.11	1	1689.11	40.95	0.0001*
BE	141.16	1	141.16	3.42	0.0745
CD	1430.72	1	1430.72	34.68	0.0001*
CE	1362.55	1	1362.55	33.03	0.0001*
DE	4.10	1	4.10	0.099	0.7549
A2	232.67	1	232.67	5.64	0.0244*
B2	2075.93	1	2075.93	50.32	0.0001*
C2	1398.17	1	1398.17	33.89	0.0001*
D2	961.06	1	961.06	23.30	0.0001*
E2	4781.79	1	4781.79	115.92	0.0001*
Lack of fit	538.56	22	24.48	0.26	0.9929
Residual	1196.30	29	41.25		
Pure error	657.74	7	93.96		

Std Dev: 6.42, Mean: 119.42, C.V (%): 5.38, R2: 0.9573, Adj R2: 0.9279, Pred R2: 0.9010, Adeq Precision: 29.441, * - Significant terms.

circular contour plots (Narang et al., 2001). Figure 1 show the interactive responses between process variables with wheat bran as carbon source, xylan as additive, peptone and yeast extract as nitrogen source and $MgSO_4$ as metal ion. The results indicate significant increase in enzyme production when wheat bran concentrations were increased from 1 to 5%. This increased activity may be due to the fact that wheat bran consists of about 40% xylan which acts as an essential substrate for xylanase enzyme (Thiago and Kellaway, 1982). Significant interaction between wheat bran and xylan may be attributed to the gene expression pattern induced by xylan suggesting inducible nature of xylanase (Parachin et al., 2009; Hiremath and Patil, 2011).

The parity plots help to determine the correlation between the predicted and the experimental values. The parity plot in Figure 2 shows a satisfactory correlation indicated by the clustering of points around the diagonal as clustering of points around the diagonal indicate good fit of model.

Experimental validation of model

From the surface plots, it was concluded that xylanase production increased with increase in the variable concentrations. The design expert model predicted the optimum concentrations of medium components as 5, 1, 1, 1 and 1 g% for wheat bran, yeast extract, xylan, $MgSO_4$ and peptone respectively for maximum xylanase production by numerical optimization step in CCD. The maximum Xylanase activity predicted with these variables at their optimum concentrations was 207.2 U/ml. Experiment in triplicates was conducted using the predicted optimized conditions by RSM for verification of model results. The experimental xylanase activity was determined to be 205.3 IU/ml which was found very close to that of predicted value.

Conclusion

In the present study, thermoalkalophillic bacteria; *Bacillus* sp. MCC 2727 was identified as an important and potent indigenous strain possessing xylanolytic characteristics. Optimization of medium components using RSM and CCD appears to be an effective and successful tool which aims at increasing enzyme productivity using time saving statistical approach. The optimum conditions

predicted by the model were wheat bran (5 g%), yeast extract and peptone (1 g% each), $MgSO_4$ (1 g%) and xylan (1 g%) which on validation produced xylanase activity of 205.3 IU/ml. These results were in good confirmation with the predicted values thus proving the accuracy of the model. Considering these results, it can be suggested that the present organism can prove to be an important source for commercial production of xylanase enzyme for applications requiring alkaline and thermophilic conditions.

Conflict of interests

The authors did not declare any conflict of interest.

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Resistance to disinfectants and antibiotics of *Pseudomonas* spp. and *Listeria* spp. biofilms on polystyrene and stainless steel

Fernanda Demoliner[1], Karen Damasceno de Souza[2], Denise Oliveira Pacheco[1], Eduarda Hallal Duval[3], Jozi Fagundes de Mello[1], Kelly Lameiro Rodrigues[1] and Eliezer Avila Gandra[4]*

[1]Postgraduate Program in Nutrition and Food (PPGNA), Faculty of Nutrition, Federal University of Pelotas (UFPel), Campus Porto, Rua Gomes Carneiro, 1 – Sala 222, CEP 96010-610, Pelotas/RS, Brazil.
[2]Graduate curse of Food Technology, Center for Chemical, Pharmaceutical and Food Sciences (CCQFA), Federal University of Pelotas (UFPel), Campus do Capão do Leao, Prédio 31, CEP 96010-900, Pelotas/RS, Brazil.
[3]Faculty of Veterinary, Federal University of Pelotas (UFPel), Campus do Capão do Leão, CEP 96010-900, Pelotas/RS, Brazil.
[4]Center for Chemical, Pharmaceutical and Food Sciences (CCQFA), Federal University of Pelotas (UFPel), Campus do Capão do Leao, Prédio 31, CEP 96010-900, Pelotas/RS, Brazil.

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The capacity of *Pseudomonas* spp. and *Listeria* spp. isolates in forming polystyrene and stainless steel biofilms was assessed and their resistance to disinfectants and antibiotics agents was verified. Isolates originated from chicken and buffalo meat cuts in abattoirs and retail outlets in the southern region of the state of Rio Grande do Sul, Brazil. Isolates which formed stainless steel biofilm were tested with regard to the activities of the disinfectant agents organic chlorine and ammonium quaternary. Isolates of *L. monocytogenes* formed polystyrene and stainless steel biofilm. Further, 32 and 72% of *Pseudomonas* spp. isolates respectively formed polystyrene and stainless steel biofilm. The disinfectant agent ammonium quaternary was more efficient than organic chlorine in the decrease of biofilms on stainless steel surfaces for *Listeria* isolates. Multi-resistance to antibiotics was high for *Listeria* spp. (94.7%) and *Pseudomonas* spp (84%). From these results, isolates from chicken and buffalo meat cuts were developers of biofilm on polystyrene and stainless steel, and resistants' to antibiotics, putting at risk consumers´ health.

Key words: Bacterial adhesion, ammonium quaternary, organic chlorine, chicken meat, buffalo meat.

INTRODUCTION

Increase in consumer demands with regard to the hygiene and sanitary conditions of meat has made producers focus on improvement in microbiological quality and food safety. Meat products are frequently

*Corresponding author. E-mail: gandraea@hotmail.com or eliezer.gandra@ufpel.edu.br. Tel: +55 5332757283; Fax: +55 5332757454.

associated with occurrences of food transmitted diseases (FTD) since meat is one of the best medium for the development of bacteria. Bacteria which develop in meat may be connected to deterioration processes or even to the transmission of diseases (Doulgeraki et al., 2012).

Bacteria of the genera *Pseudomonas* and *Listeria* may multiply and survive in fridge temperatures and may develop in cold rooms or throughout the cold chain where meat is normally stored (Jay, 2005; Todd and Notermans, 2011). *Pseudomonas* spp. are particularly deteriorating bacteria and are in the main the cause of the meat´s sensorial alterations, with a consequent decrease in shelf life (Arslan et al., 2011). Within the context of pathogenic bacteria, *Listeria monocytogenes* causes listeriosis, a serious disease with high lethality rates in risk groups (20-30%) (Lecuit and Leclercq, 2012; EFSA, 2012).

Food industries, especially meat industries, have to face several problems related to cleaning processes and sanitization of utensils and equipments. These problems are often related to the inefficiency of hygiene products and of hygiene processes in the killing or inactivating of microorganisms from the environment, with the subsequent transformation of the sites into focuses of crossed contamination. The above is due to the formation of bacterial biofilms on the equipments and in the production chain.

The formation of biofilms is enhanced in such an environment; it is actually caused by the accumulation of organic and inorganic material used by microorganisms for their fixation on the surface and the subsequent development of biofilms where communities of bacteria establish themselves and resist for long periods (Uhitil et al., 2004; Oliveira et al., 2010).

The term biofilm was created to describe the sessile form of microbial life, characterized by adhesion of microorganisms to biotic or abiotic surfaces, with consequent production of extracellular polymeric substances (Nikolaev et al., 2007; Steenackers et al., 2012). In fact, food industries, especially the processing section, are greatly impaired by biofilms which adhere to various types of surfaces especially stainless steel equipments and utensils (Marques et al., 2007; Sofos and Geornaras, 2010). Further, these bacteria are more resistant to antimicrobial activities and to disinfectant agents, causing deterioration and loss of quality in food and the dissemination of pathogens (Stepanovic et al., 2004; Hamanaka et al., 2012).

Bacterial cells in biofilms may be up to one thousand times more resistant to antibiotics than in their planktonic condition (Ouyang et al., 2012; Sharma et al., 2014). During the last decades, antimicrobial resistance, especially the multi-resistant ones, has been considered a major public health issue worldwide. The excessive and inadequate use of antibiotics may trigger the emergence of resistant bacteria favoring the dissemination of antimicrobial resistant genes in the environment (Filiousis et al., 2009; Domenech et al., 2015).

The relevance of in-depth studies on pathogenic bacteria, with special mention of *Pseudomonas* spp. and *Listeria* spp. mainly derived from animal-derived food, such as chicken and buffalo meat, should be underscored. Further, the formation process of biofilms of these bacteria in the food industry should be understood, coupled to their resistance to antibiotics and disinfectant. Preventive and corrective attitudes throughout the food chain to warrant consumers´ health will be adopted.

Current assay aimed at assessing the capacity of *Pseudomonas* spp. and *Listeria* spp. originating from chicken and buffalo meat cuts in abattoirs and retail outlets in the southern region of the state of Rio Grande do Sul, Brazil. In the formation of biofilms on polystyrene and stainless steel objects. Considering it biofilm-forming bacteria show greater resistance to antibiotics, like drugs or industrial disinfectant; the resistance to disinfectant agents used in the food industry, and to antibiotics commonly employed in people and animals also will be evaluated.

MATERIALS AND METHODS

Bacterial isolates

Current assay employed 69 bacterial isolates. Among the isolates from buffalo meat, there were fourteen isolates identified as *Listeria* species (1 isolate of *L. innocua*; 1 isolate of *L. rocourtiae* and 12 isolates of *L. grayi*) and twenty-five 25 isolates identified as *Pseudomonas* genus. Although not all species of *Listeria* used in this study are pathogenic to man as *L. monocytogenes*, some are pathogenic to animals and all have similar characteristics and the presence of a species, among chosen in this study, may indicate of the possible presence of *L. monocytogenes*. Considering the character of deterioration of the genus *Pseudomonas* in meat, and his capacity to biofilm forming, the genus identification was sufficient for selecting the isolated. They all came from a buffalo abattoir in the southern region of the state of Rio Grande do Sul, Brazil, borrowed from the bacterial bank of the Laboratory of Inspection of Animal derived Products of the Universidade Federal de Pelotas (UFPel). In the case of isolates from chicken meat, five were *L. monocytogenes* and 25 *Pseudomonas* spp. derived from the carcasses and meat cuts of chickens from a fowl abattoir and from the retail market in the southern region of Rio Grande do Sul, Brazil. Table 1 shows the isolates´ origin.

The species of *Listeria* spp. isolated from buffalo meat were confirmed in a previous study with PCR molecular tests with specific primers (data not shown), whereas the species of isolates from chicken meat were confirmed by serological tests undertaken at the Osvaldo Cruz Institution (FIOCRUZ). The genus *Pseudomonas* spp. was confirmed by biochemical phenotype tests. All isolates were frozen in a Brain and Heart Infusion Broth (BHI, Acumedia®) supplemented with glycerol (25%) till use. *Listeria* spp. isolates were recovered in Tryptone Soy Broth supplemented with 0.6% yeast extract (TSB-YE, Acumedia®) whereas *Pseudomonas* spp. isolates were recovered in a BHI broth.

Evaluation of the biofilms on polystyrene

Bacteria isolates were assessed according to their capacity for biofilm formation on polystyrene microplates following method by

Table 1. Origin of *Pseudomonas* spp. and *Listeria* spp. isolates from chicken and buffalo meat, in southern Brazil.

Isolates (n)	Origin
L. monocytogenes (3)	Chicken carcass from processing
L. monocytogenes(2)	Chicken cut from retail outlet
L. rocourtiae(1)	Buffalo carcass from processing
L. innocua(1)	Buffalo meat cut vacuum packed
L. grayi(8)	Buffalo meat cut vacuum packed
L. grayi(4)	Buffalo carcass from processing
Pseudomonas spp (14)	Chicken carcass from processing
Pseudomonas spp (11)	Chicken cut from retail outlet
Pseudomonas spp (16)	Buffalo meat cut vacuum packed
Pseudomonas spp (9)	Buffalo carcass from processing

Stepanovic et al. (2007), with modifications. Isolates were cultivated in Tryptone Soy agar (TSA, Acumedia®) at 37°C for 18 h (h) and later the bacterial concentration of the suspension was standardized by McFarland scale at 0.5, corresponding to 8 Log of Colony Forming Units per milliliter (CFU/mL). Using exactly the same volumes used with success by Stepanovic a 20 µL aliquot of the standardized suspension was distributed on microplate wells with BHI broth (180 µL, this concentration was diluted 10x on microplate) and incubated at 35°C for 24 h. Negative control comprised 200 µL of BHI broth without inoculum, whereas positive control comprised 180 µL of BHI broth and 20 µL of standardized suspension with *Staphylococcus epidermidis* (ATCC 25923) which was previously tested and classified as biofilm former. After biofilm formation, were realized modifications in relationship at protocols used by Stepanovic et al. (2007), whereas the maximum volume of each well is 200 µl the plates were washed three times with 200 µL of a sterile saline solution (NaCl 0.9%, pH adjusted to 7.0) to remove all non-adherent cells to the plate. Microplates were inverted on absorbing paper for drying. The samples were then fixed in 150 µL methanol (CH_3OH) for 20 min. After this span of time, the methanol was disposed of and the plates were kept upside down during 18 h. Adherent cells were stained with 150 µL violet crystal (0.5%) for 15 min. The stain was then removed under running water and, after drying for 3 min, 150 µL ethanol (CH_3CH_2OH) (95%) were added. Plates were kept at rest for 30 min and biofilms were counted. The optic density (OD) of the bacterial biofilm was quantified by a microplate reader (ThermoPlate®) at 450 nm.

Readings were interpreted following Stepanovic et al. (2007). Mean OD of the samples and of negative control was calculated first; then cut rate (ODc) was calculated as follows:

DOc = [average of OD negative control + (3 x standard deviation of negative control)]. Final OD rate of tested samples (DOf) was given by ODf = (mean of OD of each sample – DOc).

Samples were divided in categories, as follows:

ODf≤ODc = no biofilm former;
ODc<ODf≤2xODc = weak biofilm former;
2xODc<ODf≤4xODc = moderate biofilm former;
4xODc<ODf = Strong biofilm former.

Assessment of biofilm formation on stainless steel surface

The capacity of biofilm formation on stainless steel surfaces by bacterial isolates was assessed according to method by Rossoni and Gaylarde (2000), with modifications. Stainless steel specimens (AISI 316) measuring 7 cm x 2 cm x 0.1 cm were used. The specimens were immersed in a neutral detergent solution for 1 h; scrubbed manually with a sponge; rinsed with distilled water; sprayed with alcohol 70% and dried at 60°C. They were autoclaved at 121°C for 15 min after sterilization.

Overnight culture were prepared by seeding bacterial isolates separately in 2 mL of BHI broth and incubated at 37°C for 24 h. One milliliter of each culture was added in 40 mL of peptone water 0.85% (Silveira, 2010). Inoculum concentration added to the suspension was standardized with McFarland scale so that the bacterial concentration in 40 mL of peptone water 0.85 % would contain approximately 10^7 CFU/mL. Sterile stainless steel specimens were immersed in the bacterial suspension for 24 h at 25°C.

After immersion, the specimens were washed with 1 mL sterile distilled water to remove all weakly adhering cells. They were then scrubbed by moist swabs and immersed in test tubes with a saline solution 0.1% and homogenized in a tube shaker (Phoenix Luferco®) for 3 min (Asséré et al., 2008). Serial decimal dilutions up to 10^{-5} were performed for each sample and a 10 µL aliquot of each was seeded in TSA medium (Acumedia®) in drops (Silva et al., 2007). Plates were incubated at 37°C for 24 h for CFU counts. The microorganism *Staphylococcus epidermidis* (ATCC 25923) was the positive control. Biofilm formation on stainless steel specimens was taken into account when counts indicated a number higher than or equal to adhered 10^3 CFU/cm^2, following Wirtanen et al. (1996).

Assessing biofilm removal with disinfectant agents

A modified method by Rossoni and Gaylarde (2000) was employed to assess the removal capacity of biofilm on stainless steel plates with the disinfectant agents organic chlorine and ammonium quaternary at a concentration of 200 parts per million (ppm). Disinfectant agents and their concentration were used due to their wide use in hygiene processes in the food industry.

Induction to biofilm formation on stainless steel specimens was as described above. After the biofilm formation and the last washing, the specimens were immersed separately in flasks with organic chlorine and ammonium quaternary for 10 min. When contact time occurred, the specimens were removed from the disinfectant solution and placed in contact during 3 seconds (s) with a Tween 2% solution to neutralize the ammonium quaternary action. Each specimen was rubbed with moist swabs, followed by immersion in test tubes with a saline solution 0.1% and homogenized with a tube shaker (Phoenix Luferco®) for 3 min. Serial decimal dilutions were done for each sample; 10 µL of the suspensions were seeded in Agar TSA by drops (Silva et al., 2007); and plates were incubated at 37°C for 24 h for CFU/cm² counts. Control comprised a specimen of the material with the biofilm immersed in peptone water 0.1%, but not in contact with the disinfectant agent.

The removal of the biofilm from the stainless steel specimens was considered to have occurred when counts were less than or equal to 10^2 CFU/cm² (APHA, 1992). In this case, statistics tests were realized (analysis of variance and Tukey test at 5%).

Susceptibility to antibiotics

The susceptibility of isolates to antibiotics was tested by the disk-diffusion method following protocol by the Clinical and Laboratory Standards Institute – CLSI (CLSI 2005a). Specific antimicrobial tests were undertaken for Gram positive microorganisms in *Listeria* spp. isolates: cefepime 10 µg; rifampicin 30 µg; chloramphenicol 30 µg; vancomycin 30 µg; tetracycline 30 µg; gentamicin 10 µg;

Table 2. Classification of biofilm formers of *Listeria* spp. and*Pseudomonas* spp. isolates retrieved from chicken and buffalo meat in southern Brazil.

Isolates (n[1])	Classification about capacity biofilm forming			
	Strong	Moderate	Weak	Non-forming
L. monocytogenes[2] (5)	0	0	5	0
L. grayi[2] (12)	0	3	4	5
L. innocua[3] (1)	0	0	1	0
L. rocourtiae[3] (1)	0	0	1	0
Pseudomonas spp.[2] (25)	0	1	9	15
Pseudomonas spp.[3] (25)	0	1	5	19

[1]number of isolates; [2]originate in chicken meat; [3]originate in buffalo meat.

oxacillin 1 µg; penicillin 10 U; erythromycin 15 µg; clindamycin 2 µg; ciprofloxacin 5 µg; trimethoprim-sulfamethoxazole 25 µg. In the case of *Pseudomonas* spp. isolates, specific antibiotics for microorganisms Gram negative were tested: gentamicin 10 µg; amikacin 30 µg; trimethoprim-sulfamethoxazole 25 µg; ciprofloxacin 5 µg; meropenem 10 µg; ampicillin 10 µg; cefalotin 30 µg; cefuroxim 30 µg; amixylin 20 µg + clavulanate 10 µg; cefoxitin 30 µg; cefepime 30 µg; ceftazidime 30 µg.Standard cultures at 0.5 concentration in McFarland scale were seeded with a sterile swab in Agar Muller-Hinton (Himedia®) and disks (Multidisco, Laborclin®) impregnated with the above mentioned antibiotics were applied under the surface of the medium. After incubation at 35°C for 24 h, inhibition haloes were measured and interpreted, following CLSI (2005b).

RESULTS AND DISCUSSION

The capacity of forming biofilm on polystyrene microplate

Table 2 shows results on the classification of isolates with regard to the formation of biofilms on polystyrene plates, following Stepanovic et al. (2007). Further, 73.7 and 32% were biofilm formers, respectively for isolates *Listeria* spp. and *Pseudomonas* spp. All isolates of *L. monocytogenes* were classified weak biofilm formers. Three *L. grayi* isolates were classified moderate and four were weak biofilm formers (Table 2).

The adhesion of *Listeria* spp. to the surfaces is greatly facilitated due to its flagella, especially in the initial phases of the biofilm formation (van Houdt and Michiels, 2010). The high number of biofilm-forming *Listeria* spp. from chicken and buffalo carcasses in the processing demonstrate lack of hygiene in handling, in the sanitization of equipments and utensils and even in the conservation of the product.

Several studies have reported high biofilm formation capacity of *L. monocytogenes* on polystyrene material (Rodrigues et al., 2010; Kadam et al., 2013) and thus reveal that the material is propitious to colonization by *L. monocytogenes* biofilms.

One isolate from chicken meat and another from buffalo meat out of the evaluated 50 *Pseudomonas* spp. isolates were classified as moderate biofilm formers. Nine isolates from chicken meat and 5 from buffalo meat were classified as weak biofilm formers. The above results were corroborated by Ghadaksaz et al. (2015) who registered that 47.1% of the clinical isolates of *P. aeruginosa* were biofilm former on polystyrene. The low adhesion of *Pseudomonas* spp. isolates on polystyrene in current analysis occurred because *Pseudomonas* spp. is a hydrophobic bacterium and tends to adhere on hydrophobic surfaces rather than on hydrophilic ones (Freitas et al., 2010).

Results in current study bring great health concern since the biofilm-forming pathogenic bacteria, such as the *L. monocytogenes*, and the deterioration-causing ones, such as *Pseudomonas* spp., are a serious challenge for the food industry since they may cause crossed contamination of products, with subsequent disease transmission and decrease in shelf life (Maia et al., 2009; Giaouris et al., 2014).

The capacity of forming biofilm on stainless steel

All *Listeria* spp. isolates and 72% of *Pseudomonas* spp. in current study formed biofilms on stainless steel specimens. Further, 48 and 96% of *Pseudomonas* spp. isolates respectively retrieved from buffalo and chicken meat formed biofilm on stainless steel. Even if the number of adhered bacterial cells were less than 10^3 CFU/cm², there would still be a great risk of microbiological contamination due to microbial concentration (Wirtanen et al., 1996; Oliveira et al., 2010).

Other researchers have shown that, similar to current analysis, bacteria of the genus *Listeria* have a great ability in adhering to and forming biofilms on the surfaces of stainless steel. The bacterium proves to be a potential risk for the food industry (Moltz et al., 2005; Silva et al., 2008; Berrang et al.,2010; Oliveira et al., 2010; Bonsaglia et al., 2014).

Biofilm formation by *Pseudomonas* spp. has already been reported in previous studies. Vanhaecke et al. (1990) registered that *P. aeruginosa* isolates adhered

and formed biofilms on stainless steel surfaces within a 30-second contact. Hood and Zottola (1997) showed the formation ability of *P. fluorescens* biofilm on stainless steel with different culture media. Rossoni and Gaylarde (2000) and Rosado et al. (2006) also demonstrated the capacity of *P. fluorescens* in forming biofilms on the surface of the same material. When previously-mentioned research works performed in different places, geographically distant one from the other, and results in current study are taken into account, it may be surmised that, regardless of its origin, *Pseudomonas* spp. is capable of forming biofilms on stainless steel surfaces. The surface adhesion of *Pseudomanas* spp. may be due to flagella, since these structures give mobility to the bacterium and make it approach the substratum on the surface and, consequently, its adherence (O'Toole and Kolter, 1998).

Results obtained and the use of stainless steel in equipments and on surfaces in food processing demonstrate that *L. monocytogenes* and *Pseudomonas* spp. may contaminate food that contact the surfaces if adequate hygiene methods, coupled to adequate disinfectant agents, are not used in the food processing industries.

Biofilm removal by sanitization

Nineteen (19) biofilm formers of isolates of *Listeria* spp. and 36 isolates of *Pseudomonas* spp. on stainless steel specimens evaluated in current analysis were assessed for the removal of biofilm by organic chlorine and ammonium quaternary, two common disinfectant agents usually employed in the food industry (Table 3) (Brazil, 1988).

The disinfectant agents should remove pathogenic bacteria and reduce the number of deterioration-causing microorganisms to reasonable levels. For example, 2 CFU/cm² of mesophilic aerobic microorganisms for stainless steel surfaces at the end of the hygienization process (APHA, 1992). Taking into consideration APHA standards, 36.8% of *Listeria* spp. isolates and 77.7% of *Pseudomonas* spp. isolates adhered on stainless steel were reduced by organic chlorine. Ammonium quaternary was efficient in removing all *Listeria* spp. and 91.6% of *Pseudomonas* spp. on the surface evaluated (Table 3). Ammonium quaternary was more efficient than organic chlorine in case of isolates of *Listeria* ($p = 0.000119$), but for *Pseudomonas* isolates no significant differences between this sanitizers ($p=0.238358$). Disinfectant agents made from ammonium quaternary have a wide spectrum of activities. In fact, they change their permeability by stimulating glycolysis when in contact with the cell membrane of microorganisms and cause cell exhaustion (Andrade et al., 1996).

Studies that evaluate disinfectant agents in the killing or inactivating of *Listeria* spp. Biofilms have already been performed. However, only rare reports are extant with regard to isolated of meat cuts and to meat processing industries in south Brazil. Aarnisalo et al. (2007) and Somers and Wong (2004) showed that chlorine-based disinfectant agents were more efficient than ammonium quaternary ones in the elimination of *L. monocytogenes* adherent to stainless steel. On the other hand, Pan et al. (2006) also analyzed biofilm formation on stainless steel chips and reported the resistance of *L. monocytogenes* isolates to chlorine and ammonium quaternary. Parikh et al. (2009) assessed the efficiency of three disinfectant agents (lactic acid, sodium hypochloride and ammonium quaternary) inbiofilms composed of *L.* monocytogenes and reported that all disinfectant agents were efficacious in biofilm decrease. Ammonium quaternary was the most efficient against the developed biofilms.

Several studies analyzed disinfectant agents in the killing or inactivating of *Pseudomonas* spp. biofilms. Taylor et al. (1999) showed that the treatment of *P. aeruginosa* with chlorine-based disinfectant agent caused a decrease in biofilm within the space of 5 minutes. Wirtanen et al. (2001) reported that chlorine-based disinfectant agent was efficient in the killing or inactivating of *Pseudomonas* spp. biofilm from stainless steel surfaces, although tension-active based sanitizers were efficacious in biofilm elimination. *Pseudomonas* spp. are important bacteria in the food industry since they cause the deterioration of food products and may form biofilms in food processing equipments, albeit with great difficulty in their killing or inactivating due to their resistance to sanitizers (Zhu et al., 2014).

Susceptibility to antibiotics

The first *L. monocytogenes*strain resistant to antibiotics was isolated in 1988. Resistant strains were thenceforth detected in food, on surfaces where food is handled and in clinical samples (Gomézet al., 2014). In current study, isolates of the genus *Listeria*are highly resistant to penicillin (94.7%), followed by clindamycin (84.2%), oxacillin (73.7%) and cefepime (57.9%). Table 4 shows resistance of *Listeria* spp. isolates against 12 antibiotics that may be used in the treatment of listeriosis (Jay 2005; Arsalanet al., 2011; Allen et al., 2014; Goméz et al., 2014).

Several researchers have detected high resistance levels to penicillin in *L. monocytogenes* strains (Harakeh et al., 2009; Fallah et al., 2012), even though concern is greater when *L. monocytogenes* isolates are resistant to important antibiotics in the treatment of listeriosis. Ampicillin or penicillin with gentamicin is the first choice for the treatment of listeriosis (Charpentier et al., 1999; Conter et al., 2009).

Similar to results in current analysis, the resistance to clindamycin was also reported by Kovacevicet al. (2013),

Table 3. Efficiency of the disinfectant agents, organic chlorine and ammonium quaternary, in the removal of biofilms formed by *Listeria* spp. And *Pseudomonas* spp. retrieved from chicken and buffalo meat, in southern Brazil, on stainless steel specimens.

***Listeria* isolates**	**Bacterial cells adhered on stainless steel (CFU/cm²)**	**Organic chlorine (CFU/cm²)**	**Ammonium quaternary (CFU/cm²)**
L. monocytogenes	$2.7x10^4$	$2.1x10^3$	-
L. monocytogenes	$5.7x10^5$	-	-
L. monocytogenes	$1.2x10^4$	$1x10^2$	-
L. monocytogenes	$1x10^6$	-	-
L. monocytogenes	$5.1x10^6$	$8.5x10^4$	-
L. innocua	$4.2x10^2$	-	-
L. rocourtiae	$5.1x10^5$	$2.3x10^4$	-
L. grayi	$6.4x10^6$	$1.2x10^5$	-
L. grayi	$2.1x10^5$	$1.9x10^4$	-
L. grayi	$3.8x10^5$	$1.2x10^4$	-
L. grayi	$6.4x10^4$	$5.9x10^4$	-
L. grayi	$1.9x10^6$	-	-
L. grayi	$2.1x10^5$	$1x10^5$	-
L. grayi	$8.1x10^5$	$2.7x10^4$	-
L. grayi	$4.2x10^5$	-	-
L. grayi	$3.8x10^4$	-	-
L. grayi	$1.2x10^5$	$5.1x10^4$	-
L. grayi	$2.3x10^4$	$6.4x10^3$	-
L. grayi	$1.9x10^4$	$8.5x10^3$	-
Pseudomonas spp.	$1.4x10^5$	-	-
Pseudomonas spp.	$4.8x10^5$	-	-
Pseudomonas spp.	$1.4x10^5$	$6.4x10^2$	-
Pseudomonas spp.	$2.5x10^7$	$4.2x10^3$	-
Pseudomonas spp.	$2.1x10^5$	-	-
Pseudomonas spp.	$2.1x10^6$	-	-
Pseudomonas spp.	$1.6x10^6$	-	-
Pseudomonas spp.	$2.3x10^5$	$2.1x10^2$	-
Pseudomonas spp.	$3.8x10^5$	-	-
Pseudomonas spp.	$1.4x10^6$	$2x10^2$	-
Pseudomonas spp.	$1.2x10^7$	-	-
Pseudomonas spp.	$3.8x10^3$	-	$2x10^3$
Pseudomonas spp.	$6.4x10^6$	-	-
Pseudomonas spp.	$4.5x10^6$	$2.1x10^2$	-
Pseudomonas spp.	$6.4x10^5$	-	-
Pseudomonas spp.	$1.2\ x10^6$	-	-
Pseudomonas spp.	$4.8\ x10^6$	-	-
Pseudomonas spp.	$2.3\ x10^6$	-	-
Pseudomonas spp.	$4.8x10^6$	-	-
Pseudomonas spp.	$3.6x10^4$	-	-
Pseudomonas spp.	$1.6\ x10^6$	-	-
Pseudomonas spp.	$2.1x10^5$	-	-
Pseudomonas spp.	$2.9\ x10^6$	-	-
Pseudomonas spp.	$1.6\ x10^6$	-	-
Pseudomonas spp.	$1.6x10^4$	-	-
Pseudomonas spp.	$8.3x10^4$	-	$8.5x10^3$
Pseudomonas spp.	$8.3x10^5$	-	-
Pseudomonas spp.	$3.8x10^5$	-	-

Table 3. Contd.

Listeria isolates	Bacterial cells adhered on stainless steel (CFU/cm²)	Organic chlorine (CFU/cm²)	Ammonium quaternary (CFU/cm²)
Pseudomonas spp.	$8.2x10^4$	-	-
Pseudomonas spp.	$3.8x10^5$	-	-
Pseudomonas spp.	$1.4x10^4$	-	-
Pseudomonas spp.	$5.3x10^4$	$2.1x10^3$	-
Pseudomonas spp.	$5.3 x10^4$	-	-
Pseudomonas spp.	$3.4 x10^4$	-	-
Pseudomonas spp.	$1.4x10^5$	$2.1x10^3$	-
Pseudomonas spp.	$1.4x10^5$	$3.8x10^3$	$1.7x10^3$

-: Bacterial absence.

Table 4. Resistance to antibiotics of *Listeria* spp. isolated retrieved from chicken and buffalo meat, in south Brazil.

Isolate (n)	Number of isolates of the *Listeria* spp. species											
	CPM (R/I)	RIF (R/I)	CLO (R/I)	VAN (R/I)	TET (R/I)	GEN (R/I)	OXA (R/I)	PEN (R/I)	ERI (R/I)	CLI (R/I)	CIP (R/I)	SUT (R/I)
Chicken												
L. monocytogenes (5)	1/2	-	-	-	-	1/0	3/1	4/0	0/3	3/1	1/0	-
Buffalo												
L. grayi (12)	8/3	0/1	-	-	2/3	0/6	10/1	12/0	2/4	11/1	0/1	-
L. innocua (1)	1/0	0/1	-	1/0	1/0	-	0/1	1/0	0/1	1/0	-	-
L. rocourtiae (1)	1/0	-	-	-	1/0	0/1	1/0	1/0	0/1	1/0	-	-
Total	11/5	0/2	0	0	4/3	1/7	14/3	18	2/9	16/2	1/1	0

CPM: cefepime 10 µg; RFI: rifampicin 30 µg; CLO: chloramphenicol 30 µg; VAN: vancomycin 30 µg; TET: tetracycline 30 µg; GEN: gentamicin 10 µg; OXA: oxacillin 1 µg; PEN: penicillin 10 U; ERI: erythromycin 15 µg; CLI: clindamycin 2 µg; CIP: ciprofloxacin 5 µg; SUT: trimethoprim-sulfamethoxazole25 µg; (R/I), where R = Resistance, and I = Intermediary resistance.

where 33% of *Listeria* spp., derived from fish, meat and processing factories, were resistant to clindamycin. Gómez et al. (2014) also registered clindamycin-resistant isolates, 35% *L. monocytogenes* and 46.2% *L. innocua*, retrieved from meat products and from the processing environment. According to Harakehet al. (2009), resistance of *L. monocytogenes* to penicillin and clindamycin may have been caused by drug excess in veterinary medicine.

All isolates tested in current analysis are sensitive to chloramphenicol and only one was resistant to gentamicin and ciprofloxacin. Similar results were reported in studies by Doménech et al. (2015) in which all *L. monocytogenes* isolates from ready-made food were sensitive to the three antibiotics. Gómez et al. (2014) also detected sensitivity to chloramphenicol in all *L. monocytogenes* isolates and in 99.2% of *L. innocua*. Kovacevicet al. (2013) reported sensitivity in all *Listeria* spp. isolates to gentamicin. The high sensitivity of isolates to gentamicin may be due to the fact that it is neither an antimicrobial agent usually used in veterinary therapy nor a growth enhancer in beef cattle (Harakehet al., 2009).

Sensitiveness to trimethoprim-sulfamethoxazole and vancomycin occurred in all isolates in current study. Yan et al. (2010) reported few *L. monocytogenes* isolates retrieved from food which were resistant to trimethoprim-sulfamethoxazole (Sulfazotrim) and vancomycin. However, Kovacevic et al. (2013) and Korsak et al. (2012) reported all isolates as sensitive to vancomycin. Doménech et al. (2015) registered that all *L. monocytogenes* isolates derived from pork sausages were resistant to trimethoprim-sulfamethoxazole. These results are highly relevant since theantimicrobial agent ranks second in the treatment for listeriosis, especially in patients allergic to penicillin (Pesaventoet al., 2010). According to Harakeh et al. (2009), vancomycin is the last ranking in treatment for infections with listeriosis in humans.

In general terms, *L. monocytogenes*, retrieved from chicken meat on the retail market, was the only isolate sensitive to all the antibiotics under analysis, although

Table 5. Resistance of *Pseudomonas* spp. isolates retrieved from chicken and buffalo meat in southern Brazil, to antibiotics.

Isolate (n)	Number of *Pseudomonas* spp. isolates											
	GEM (R/I)	AMI (R/I)	SUT (R/I)	CIP (R/I)	MER (R/I)	AMP (R/I)	CFL (R/I)	CRX (R/I)	AMC (R/I)	CFO (R/I)	CPM (R/I)	CAZ (R/I)
Chicken												
Pseudomonas spp. (50)	0/1	-	-	-	25/0	1/5	0/13	13/7	10/11	13/8	-	-
Buffalo												
Pseudomonas spp. (50)	-	-	1/2	0/2	24/1	6/1	1/4	12/2	3/15	11/0	-	-
Total	0/1	0	1/2	0/2	49/1	7/6	1/13	25/9	13/26	24/8	0	0

GEM: Gentamicin 10 µg; AMI: amikacin 30 µg; SUT: sulfazotrim 25 µg; CIP: cipofloxacin 5 µg; MER: meropenem 10 µg; AMP: ampicillin 10 µg; CFL: cefalotin 30 µg; CRX: cefuroxime 30 µg; AMC: amixillin+clavulanate 30 µg; CFO: cefoxitin 30 µg; CPM: cefepime 30 µg; CAZ: ceftazidime 30 µg; (R/I) where R = Resistance; I = Intermediary resistance.

21% of isolates tested were resistant to two antibiotics and 73.7% were resistant to three to five antibiotics. Isolates resistant to two or more antibiotics, totally 94.7%, were classified as multi-resistant. In fact, multi-resistance is not restricted to these isolates in southern Brazil since several studies have detected *Listeria* spp. isolates, multi-resistant to antibiotics, as a worldwide issue (Conter et al., 2009; Yan et al., 2010; Pesavento et al., 2010; Fallah et al., 2012; Goméz et al., 2014). *Listeria* spp. multi-resistant isolates against antibiotics usually used in the treatment of human listeriosis are a grave issue in public health due to a more difficult therapy especially for people in risk groups, involving elderly people, children, pregnant women and immunocompromised people (Goméz et al., 2014).

Table 5 shows the susceptibility of *Pseudomonas* spp. isolates to the 12 antibiotics tested, used for treatment of infections mainly caused by *P. aeruginosa* (Tassios et al., 1998; Jeukens et al., 2014). The highest resistance rate occurred for meropenem, with all isolates derived from chicken meat and 96 % from buffalo meat. There was no resistance in chicken and fish isolates to antibiotics among the *P. aeruginosas* strains belonging to the carbapenemclass (imipenemand meropenem), evaluated by Maia et al. (2009). In fact, they are used for multi-resistant isolates. Results in current analysis are grave since meropenem is an effective antimicrobial agent in the treatment of infections caused by Gram negative bacteria (Gales et al., 2002).

In the case of multi-resistance, 92% of isolates retrieved from chicken meat and 76% of isolates retrieved from buffalo meat were resistant to more than two antibiotics. Multi-resistant increase to antibiotics in Gram negative bacteria and specifically in *P. aeruginosa* indicate a reduced availability of effective agents for treatments in infections caused by this bacterium. Resistance increase to antibiotics and the potential for global dissemination of resistance genes to pathogen bacteria have become a world health issue for human and veterinarian medicine (Arslan et al., 2011; Sharma et al., 2014). The excessive use of antibiotics in veterinary medicine may be related to pathogens derived from the food chain resistant to antibiotics used by humans (Wang et al., 2007). It is highly important in the context of resistance and multi-resistance to anti-microbial agents to control and monitor the correct employment of these antibiotics in the treatment of people and in veterinary medicine to decrease the transmission of resistance in the food chain.

The testing was performed considering the hypothesis that biofilm-forming bacteria show greater resistance to antimicrobial agents, like drugs, antibiotics or industrial disinfectant. All isolates, who underwent removal test by sanitizers, have formed biofilm on stainless steel. However, not all isolates were resistant antibiotic. Among the *Listeria* isolates, seven *L. gray* which were resistant to organic chlorine were also resistant to two types of antibiotics; One *L. innocua* was resistant the organic chlorine and also to two types of antibiotics; One *L. roucotiae* which was resistant to organic chlorine was also to five types of antibiotics. Among the three *L. monocytogenes*, which were resistant to organic chlorine, two of these were resistant to three types of antibiotics and one was sensible to all antibiotics. In the case of *Pseudomonas* spp isolates, nine isolates were resistant to organic chlorine; eight of these were also resistant at least to two antibiotics. Three isolates, which were resistant to the ammonium quaternary, were also resistant to two types of antibiotics. In this study, it not possible establish a clear relationship positive or negative between the antibiotics and disinfectant resistances verified.

Conclusions

Results demonstrate the importance of control of microbial biofilms in the meat industry since current analysis revealed that isolates of *Listeria* spp. and *Pseudomonas* spp. Derived from chicken and buffalo

meat were capable of forming biofilms on polystyrene and stainless steel specimens.

The activities of the two disinfectant agents, organic chlorine and ammonium quaternary, were efficient in removing biofilms of *Listeria* spp. and *Pseudomonas* spp. on stainless steel specimens. The second agent was more efficient for *Listeria* spp. So that biofilm risk may be minimized, it is important that the food industry employs control strategies, such as efficient hygiene process that comprises correctly all the stages of cleaning and disinfectant, with recommended products and at the best concentrations for the elimination of microorganisms.

This study identified multi-resistance and resistance to antibiotics in several *Listeria* spp. and *Pseudomonas* spp. isolates.

Conflict of interest

The authors have not declared any conflict of interests.

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